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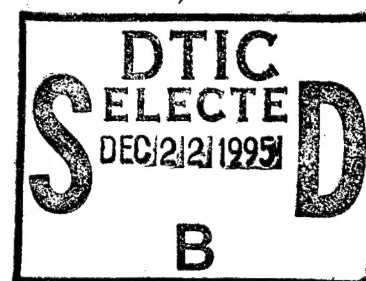
TITLE: Studies on Platelet-Derived Growth Factor Beta-Receptor
and Hepatocyte Growth Factor Receptor c-met in Paracrine
Interactions in Human Breast Cancer

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REPORT DATE: September 1995

TYPE OF REPORT: Annual



PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

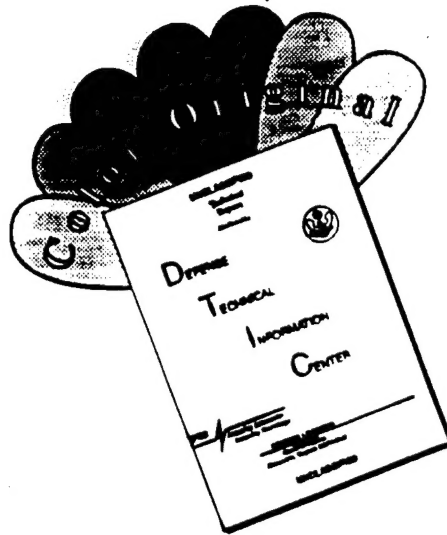
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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 1995	3. REPORT TYPE AND DATES COVERED Annual 1 Sep 94 - 31 Aug 95	
4. TITLE AND SUBTITLE Studies on Platelet-Derived Growth Factor Beta-Receptor and Hepatocyte Growth Factor Receptor c-met in Paracrine Interactions in Human Breast Cancer		5. FUNDING NUMBERS DAMD17-94-J-4407	
6. AUTHOR(S) Dr. Bruce E. Elliot			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Queens University Kingston, Ontario, Canada K7L 3N6		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Platelet-derived growth factor (PDGF) and hepatocyte growth factor (HGF) are key regulators of breast stromal-epithelial interactions. We examined the expression of PDGF β -receptor, HGF receptor/Met and the corresponding ligands in primary human breast cancer using <i>in situ</i> hybridization and immunohistochemical techniques. The results showed that PDGF β -receptor was localized in smooth muscle-actin-positive cells and vascular endothelial cells in the periepithelial stroma, but not in the epithelial component of ductal carcinoma <i>in situ</i> . These findings support the notion that PDGF BB, which is produced by breast carcinoma cells, functions in the paracrine stimulation of the stroma by adjacent carcinoma cells. In contrast, HGF receptor/Met was exclusively expressed on nonmalignant and malignant epithelial cells. However, HGF mRNA was expressed in both stromal cells and in carcinoma cells in regions of invasive cancer; these results suggest that both paracrine and autocrine stimulation by HGF of mammary carcinoma cells can occur. Our hypothesis is that co-expression of HGF and HGF receptor/Met results in the establishment of an HGF autocrine loop which provides a selective advantage for autonomous growth and metastasis of mammary carcinoma cells. To further investigate the possible role of an HGF autocrine loop in the progression of breast cancer, we are currently examining expression and function of HGF in human and murine primary breast carcinomas and carcinoma cell lines.			
14. SUBJECT TERMS Platelet-derived growth factor; hepatocyte growth factor; stromal cells; breast cancer; metastasis		15. NUMBER OF PAGES 112	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION Unclassified		18. LIMITATION OF ABSTRACT Unlimited	
19. SECURITY CLASSIFICATION Unclassified		20. LIMITATION OF ABSTRACT Unlimited	
21. SECURITY CLASSIFICATION Unclassified		22. LIMITATION OF ABSTRACT Unlimited	

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Bruce N. Elliott Oct 4, 1995
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of Annual Report for Grant #DAMD17-94-J-4407

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INTRODUCTION:

Breast cancer affects 1 in 9 women, and approximately 1 in 3 women who have breast cancer will die from metastases. The growth and spread (metastasis) of breast cancer is influenced by surrounding normal tissue as well as progressive changes in malignant cells. The development of reliable diagnosis and treatment requires an understanding of the cell biology and molecular mechanisms involved in tumor growth and metastasis.

Two growth factors, platelet-derived growth factor (PDGF) and hepatocyte growth factor (HGF) have been shown to act as key regulators of many stromal-epithelial interactions. PDGF, produced by many breast carcinoma cells (1-3), can stimulate proliferation (4) and angiogenesis (5) of surrounding stromal cells. Unlike PDGF, HGF is produced by most stromal cell types (6), and can stimulate normal development of breast epithelium (7) and invasiveness of carcinoma cells (8). Thus, these two growth factor pathways may function in a reciprocal manner. The overall aim of this programme is to identify cell types expressing PDGF, HGF and their corresponding receptors, in human breast cancer and to assess their regulatory role in breast cancer progression.

PDGF BB and PDGF β -receptor: PDGF BB, which is upregulated in the majority of breast cancer tissues (1-3) and in breast carcinoma cell lines (5,9), is one important candidate molecule in the paracrine stimulation of the stroma by carcinoma cells. PDGF BB has been shown to stimulate a variety of functions including growth (4), chemotaxis (10), and angiogenesis (5) in connective tissue cells. PDGF is composed of two chains, A and B, held together by disulfide bonds and present in three dimeric forms AA, AB and BB (11,12). The dimers bind to two separate receptor types: α and β (11,12). The α -receptors bind to all three isoforms of PDGF, while the β -receptors have a very high affinity for PDGF BB, bind PDGF AB to some extent, but do not bind PDGF AA at all. The β -receptor has a ligand activated protein kinase (13), the receptor being autophosphorylated in response to the appropriate ligand. Each PDGF chain is coded by a distinct gene. The gene encoding PDGF B has been identified as the human analogue of the v-sis oncogene (12,14,15). Transfection of the PDGF B gene into fibroblasts showed a higher transforming activity, compared to the PDGF A gene (16). PDGF β -receptor mRNA and protein have been demonstrated on most stromal cell types including fibroblasts (17), smooth muscle cells (10), endothelial cells (18), and pericytes (19). Recently, some breast carcinoma cell lines (20), as well as breast cancer tissues (3,21,22) have also been shown to express PDGF β -receptors. However, it is not known what cell types in primary human breast cancer express PDGF β -receptors.

HGF and HGF receptor/Met: HGF has been shown to stimulate a variety of cell functions including proliferation in some cells (23), differentiation (24), cell motility (8), invasion (8), and angiogenesis (25). In addition, HGF can stimulate mesenchyme-epithelial cell transition (26), a process that contributes to altered differentiation and possible recruitment of carcinoma cells from mesenchymal tumors. HGF is synthesized as a pre-pro-HGF of 728 amino acid residues. Following the co-translational removal of the N-terminal signal sequence, HGF is secreted as an inactive single-chain pro-HGF protein of 105 KDa (27). Pro-HGF is cleaved by serine proteinases at Arg494-Val495, and is converted to a heterodimeric mature HGF molecule consisting of disulfide-linked α and β chains of 463 and 234 amino acid residues, respectively (27). The maturation of pro-HGF is required for the biological activities of HGF. Secreted HGF normally remains as the single-chain precursor form,

probably associated with the extracellular matrix in the producing tissues. During development, tissue repair, and organ regeneration certain serine proteinases such as Factor XIIa (28), urokinase (29) and tissue-type plasminogen activator (30) are activated and can convert single-chain pro-HGF to the biologically active heterodimer.

The met proto-oncogene product, a member of the tyrosine kinase receptor family, has been identified as the HGF receptor (31). The HGF receptor/Met is a heterodimer, composed of a 50 KDa α chain, disulfide-linked to a 145 KDa β chain. The α chain is exposed at the surface; the β chain has a transmembrane domain and a tyrosine kinase domain on the cytoplasmic side. HGF receptor/Met is expressed in many types of cells, especially those of epithelial origin (32). In many human carcinoma cell lines, met is amplified (33), over-expressed (34-37), or constitutively activated (36). Activation of HGF receptor/Met stimulates most HGF-induced functions (38,39), however which HGF functions are involved in producing the malignant phenotype are not known.

In the present project, we examined the expression of PDGF β -receptor, HGF receptor/Met and the corresponding ligands in primary human breast cancer. The results showed that PDGF β -receptor was localized in the periepithelial stroma but not in the epithelial component of breast carcinoma *in situ*. These findings support the notion that PDGF BB, which is produced by breast carcinoma cells, functions in the paracrine stimulation of the periepithelial stroma by the tumor cells. In contrast, HGF receptor/Met was exclusively expressed on nonmalignant and malignant epithelial cells, but not stromal cells. However, HGF mRNA was expressed in both stromal regions and in regions of invasive carcinoma, raising the possibility that both paracrine and autocrine stimulation by HGF of mammary carcinoma cells could occur. Based on these findings, our hypothesis is that co-expression of HGF and HGF receptor/Met results in the establishment of an HGF autocrine loop which provides a selective advantage for autonomous growth and metastasis of mammary carcinoma cells. To further investigate the possible role of an HGF autocrine loop in the progression of breast cancer, we are examining the expression and function of HGF in human and murine breast carcinoma cell lines.

BODY:

During the past year we have obtained results addressing Objectives 1 and 2 of US Army Materiel and command grant DAMD 17-94-J-4407: the expression of PDGF β -receptor, HGF receptor/Met and corresponding ligands in primary human breast cancer tissue. In addition, we have begun to examine the expression and function of HGF in various human and mouse nonmalignant and malignant breast epithelial cell lines. These results are summarized below and in the attached manuscripts.

Expression of platelet-derived growth factor β -receptor in the periepithelial stroma of human breast cancer: In the present study, the tissue localization of PDGF β -receptor expression was studied in human breast carcinoma and non-malignant breast tissues using both immunofluorescence and immunoperoxidase staining techniques. We examined a total of 29 cases of infiltrating ductal carcinomas, which showed both carcinoma *in situ* and invasive components. PDGF β -receptor staining was localized in the periepithelial stroma, and was particularly intense in regions immediately adjacent to carcinoma *in situ* components. A diffuse low level of PDGF β -receptor staining was seen

throughout the stroma of 8 non-malignant breast tissues as well as non-malignant regions of tumor tissues. Image analysis was used to assess the coincidence of staining of PDGF β -receptor with epithelial or stromal cells in 13 of the 29 tumor tissues studied. Less than 5% of malignant ductal epithelium or myoepithelium showed PDGF β -receptor staining. Analysis with stromal cell type-specific markers indicated significant localization of PDGF β -receptor primarily with α -smooth muscle actin staining cells (32%) and vascular endothelial cells (41%) in the periepithelial stroma. PDGF β -receptor positivity was strongly associated with basement membrane surrounding regions of carcinoma *in situ*, but was less intense in regions of invasive carcinoma where basement membrane was degraded. The absence of PDGF β -receptors on carcinoma cells and their presence in the surrounding stroma suggests a paracrine stimulation of adjacent stromal tissue by malignant epithelial cells in human breast tumors. A manuscript of this work is currently being reviewed for publication in *Clin. Cancer Res.* (See App. I).

Co-expression of hepatocyte growth factor and receptor/Met in human breast carcinoma:

Expression of HGF and HGF receptor/Met mRNA were examined using non-isotopic *in situ* hybridization (ISH) in a spectrum of benign and malignant human breast tissues. mRNA for both HGF receptor/Met and HGF was detected in benign ductal epithelium. Epithelial expression of HGF mRNA was particularly intense in regions of ductal epithelial hyperplasia. Positive expression of HGF (but not HGF receptor/Met) mRNA was also found in adipocytes, endothelial cells, and to varying degrees in stromal fibroblasts. In 12 of 12 cases of ductal carcinoma *in situ* and infiltrating ductal carcinoma, carcinoma cells showed a heterogeneous pattern of expression for both HGF receptor/Met and HGF mRNA. In infiltrating ductal carcinomas, intense expression of HGF receptor/Met mRNA was not restricted to ductular structures, but was also seen in non-duct-forming carcinoma cells. The same zones of the tumors (most commonly at the advancing margins) that expressed strongly HGF receptor/Met mRNA often were also strongly positive for HGF mRNA, suggesting a possible autocrine effect. The expression pattern of HGF receptor/Met protein in 25 cases including the same series of tissues used for ISH analysis was similar to that of HGF receptor/Met mRNA, as determined by an immunoperoxidase technique. The finding that HGF receptor/Met is expressed by both benign and malignant epithelium, and is not restricted to duct-forming structures, suggests that although the potential for HGF/HGF receptor binding is maintained in malignancy, the response to ligand binding at the level of the receptor, or the cellular response to receptor activation may change at some point during progression. See Tuck *et al.* *Am. J. Pathol.* In press, 1995 (Appendix II).

Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma:

The above results suggest that autocrine production of HGF by malignant epithelial cells may be associated with the malignant phenotype of breast carcinoma cells. We have previously demonstrated that HGF is a potent mitogenic factor for murine mammary carcinoma (SP1) cells *in vitro*. We report here an autocrine HGF loop in SP1 cells. HGF receptor/Met is expressed in SP1 cells and is constitutively tyrosine-phosphorylated. The phosphorylation of HGF receptor/Met is inhibited when cells are exposed to suramin or anti-HGF IgG. This finding suggests that constitutive tyrosine-phosphorylation of HGF receptor/Met is sustained by an extracellular factor, most likely HGF. Using northern blot and western blot analysis, we detected expression of a 6 kb HGF mRNA in SP1 cells and an 85 KDa HGF protein in SP1 conditioned medium, respectively. *In vitro* translation of mRNA from SP1 cells and metabolic labeling confirmed expression and synthesis of HGF by SP1 cells.

SP1 cells also invade through matrigel-coated transwell membranes in an in vitro invasion assay and invasion of these cells was inhibited by neutralizing anti-HGF IgG. In addition, SP1 conditioned medium induced scatter activity of Madin-Darby canine kidney epithelial cells, and this activity was inhibited by neutralizing anti-HGF IgG. We have also shown that several signaling molecules including phosphatidylinositol 3-kinase, Src, focal adhesion kinase, and phospholipase C- in SP1 cells are constitutively tyrosine-phosphorylated, suggesting that co-expression of HGF and HGF receptor/Met may in part contribute to sustained tyrosine-phosphorylation of these cytoplasmic proteins in SP1 cells. Our observations in the SP1 model suggest that HGF contributes to growth and invasive phenotypes of mammary carcinomas via both paracrine and autocrine mechanisms. This work has been accepted with revisions for publication in *Cell Growth and Differ.* (1995) (See Appendix III).

Examination of an HGF autocrine loop in human breast carcinoma cells (In progress):

Preliminary results showed immunoreactive HGF protein associated with carcinoma cells in human breast tissue sections (Tuck *et al.*, unpublished result). However, exogenous HGF protein could be bound to, or endocytosed by, these carcinoma cells. Furthermore, it is not known from these studies whether post-translational processing of pro-HGF occurs in benign or malignant epithelial cells from primary breast tissues. We are therefore examining the expression of HGF mRNA and protein in primary human breast tissues and in newly-established nonmalignant and malignant epithelial cell lines. The maturation state of HGF produced by epithelial cells will be assessed by both biochemical and functional approaches. In addition, activation of the HGF receptor/Met and HGF-dependent cell functions will be assessed to determine the presence of a possible HGF autocrine loop.

To detect expression of putative HGF mRNA and protein in nonmalignant and malignant breast epithelial cells, we have initiated RNase protection (40) and western blot analyses of carcinoma cells purified from human breast tumor tissues and various newly derived non-malignant and malignant breast epithelial cell lines (see attached list). In collaboration with Dr. O. Petersen (Denmark, letter attached), we are examining HGF and HGF receptor/Met expression in isolated pure populations of stromal and epithelial cells from human breast tumor tissues (41). These purified populations of stromal and breast epithelial cells have proven to be excellent in vitro correlates for the study of stromal-epithelial cell interactions in human breast (41). In this way we will confirm whether HGF and HGF receptor/Met are expressed in stromal and epithelial cells freshly isolated from human breast without cell culture.

In addition to primary human breast tissue, we are also examining various nonmalignant and malignant breast epithelial cell lines (see list attached). One murine mammary carcinoma, SP1, expressed a 6 kb HGF mRNA, and secreted an 82 KDa HGF protein, corresponding to the reported sizes of HGF mRNA and mature protein, respectively (27,42) (Appendix III). A similar approach will be used to examine HGF and HGF receptor/Met expression in two mammary epithelial cell lines and corresponding malignant cell lines derived from them: MCF10A (43) (human, obtained from F. Miller) and TM3 (mouse, obtained from D. Medina) cells. MCF10A cells were transfected with the Ha-Ras oncogene to yield a cell line, MCF10A1neoT, which forms tumors in nude mice (designated MCF10A-TB3) (see attached list). Two other newly established human breast carcinoma cell lines are also being studied (WO-E, EL-E, obtained from Dr. B. Campling, Dept. Oncology, Queen's U.). We have recently shown that MCF10A-TB3, EL-E, but not WO-E carcinoma cells, secrete mature HGF protein and express HGF receptor/Met as shown in Figure 1.

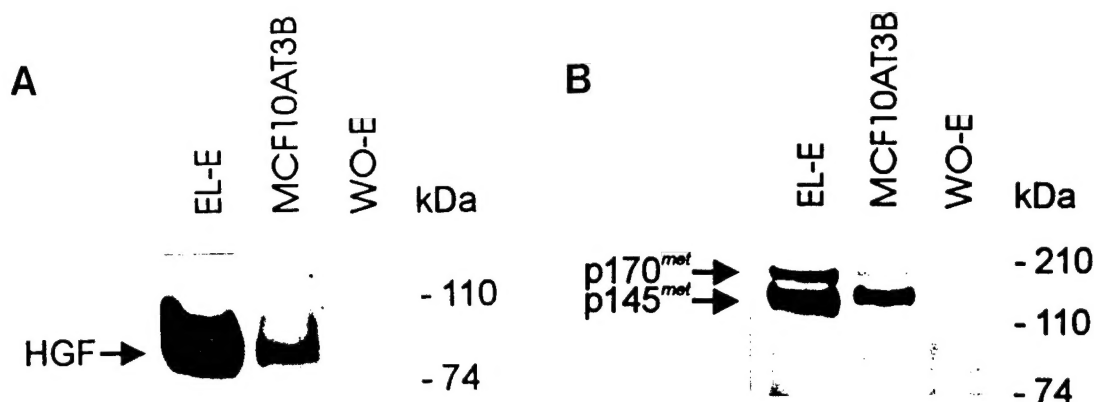


Figure 1: Detection of HGF and HGF receptor/Met from human breast carcinoma cells:

Panel A: CM from three breast carcinoma cell lines were concentrated (30x), and HGF was affinity-purified with Cu(II)-coupled beads (Rahimi and Elliott, manuscript submitted). The membrane was probed with anti-human IgG (Genentech Inc.). Lane A, EL-E; Lane B, MCF10A-TB3; Lane C, WO-E.

Panel B: Cell lysates from the same cell lines were immunoprecipitated and blotted with anti-human Met IgG (obtained from Dr. M. Park). Lane A, EL-E; Lane B, MCF10A-TB3; Lane C, WO-E.

Biosynthetic labeling experiments are in progress to confirm that HGF is synthesized by these cells. The biological activity of the putative HGF will be assessed using a scatter assay with MDCK cells, and the HGF-dependency of this activity will be confirmed by neutralizing anti-HGF IgG (Appendix III). The MCF10A and TM3 series of mammary epithelial cell lines allow comparison of HGF expression in the same cell lineage at nonmalignant and malignant stages of growth.

CONCLUSIONS:

In the present study, we examined the presence and possible involvement of PDGF and HGF and their corresponding receptors, in human breast cancer. Our results support paracrine stimulation by PDGF BB, which is secreted by breast carcinoma cells, of the periepithelial stroma. In contrast, both paracrine and autocrine stimulation of breast carcinoma cells by HGF was evident.

PDGF BB and PDGF β -receptor: We showed that PDGF β -receptor expression is essentially absent in both non-malignant and malignant epithelial cells. However, PDGF β -receptor expression was intense and localized in the periepithelial stroma of ductal carcinoma *in situ* (DCIS); whereas, non-malignant tissue displayed a more diffuse weaker expression of PDGF β -receptors. These findings confirm the recent report by Coltrera *et al.* (3) that PDGF β -receptor is expressed in fibroblastic stroma cells in non-malignant breast and DCIS. We have further demonstrated that the intense PDGF β -receptor staining in malignant breast tissue was co-localized with α -smooth muscle actin (SMA) staining cells and endothelial cells near the basement membrane surrounding malignant ducts, but not with myoepithelia. These results imply a paracrine stimulation by malignant epithelia of specific cell types in adjacent stroma.

HGF and HGF receptor/Met: In contrast to PDGF β -receptor, expression of HGF receptor/Met mRNA was detected in both benign and malignant ductal epithelium. These findings are consistent with the demonstration by Tsarfaty *et al.* (26) of HGF receptor/Met protein expression in similar tissues. However, in contrast to what has been described previously (26), we have found that expression of HGF receptor/Met mRNA and protein levels are not decreased uniformly in malignant epithelium, and expression of HGF receptor/Met is not limited to cells lining ductules or tubules. In fact, we have found that both *in situ* and invasive ductal carcinomas of all grades show a heterogeneous pattern of HGF receptor/Met expression. Individual malignant cells or groups of cells of poorly differentiated infiltrating ductal carcinoma (IDC) were commonly found to stain strongly for both HGF receptor/Met mRNA and protein. Level of expression of HGF receptor/Met in itself is thus not necessarily associated with degree of ductal differentiation.

We detected HGF mRNA expression in benign and malignant mammary ductal epithelium, as well as variable expression in stromal cells (fibroblasts, adipocytes and endothelial cells), in a manner similar to that described by Wang *et al.* (44). Zonal co-expression of HGF ligand and receptor mRNA was found in both benign and malignant epithelium, and would suggest the possibility of an HGF autocrine loop. This finding is consistent with recent evidence that HGF receptor/Met expression may be upregulated by HGF (45). A similar autocrine loop effect for HGF and HGF receptor/Met in malignant (and some non-malignant, proliferative) epithelial cells has been described previously in other systems (46,47). The higher levels of co-expression of ligand and receptor observed in ductal epithelial hyperplasia suggests that HGF may act in an autocrine role in non-neoplastic ducts as well, especially those undergoing benign proliferation. In the malignant situation, a normal reactive mechanism may be altered as suggested above, such that autocrine stimulation itself is no longer properly regulated, or the response to autocrine stimulation is altered, with the end result of invasion and metastasis ("malignant conversion") rather than morphogenesis and ductular differentiation ("tumor suppression"). Also in keeping with this hypothesis is the finding that in the majority of the tumors examined, highest levels of expression of both HGF and HGF receptor/Met mRNA were found at the advancing margins of the tumor, where active invasion is taking place. This pattern of expression has been reported for other peptides, e.g. transforming growth factor β (48), platelet-derived growth factor β receptor (49), cathepsin B (50), and plasminogen activator (51), which may be involved in invasiveness at the tumor front. Interestingly, urokinase-type plasminogen activator is also an activator of HGF (29).

Future Studies: The next stage of this project will focus on the issue of autocrine expression of HGF in breast carcinoma cells and the relevance of an HGF autocrine loop in breast cancer progression. Two main approaches are in progress. **First**, we will characterize putative HGF and HGF receptor/Met expressed in some human and murine mammary carcinoma cell lines. Further studies are in progress to examine the function of putative HGF produced by mammary carcinoma cell lines. Since HGF requires post-translational processing to produce biologically active HGF, it is possible that some carcinoma cells may express HGF mRNA, but do not express the necessary proteinases for maturation of HGF. We are therefore currently examining the HGF-activating capacity of CMs from various human mammary carcinoma cell lines (e.g. EL-E and MCF10A-TB3) which secrete mature HGF. It is also possible that HGF receptor/Met is expressed in these carcinoma cells but may be dysfunctional in some way. Experiments are therefore in progress to assess the activation of HGF receptor/Met in breast

tumor tissues and carcinoma cell lines, and to correlate the level of receptor activation with endogenous production of HGF. HGF-dependent functions in non-malignant and malignant epithelial cells will also be assessed. Some of the functions to be examined are: cell proliferation (cyclins A (52), D1 (53,54) and E(55)), differentiation (E-cadherins (56)), and cell motility and invasiveness (Appendix III). Where possible, human breast tumor tissues (57) or carcinoma cells purified from breast tissues with known patterns of HGF and HGF receptor/Met, expression will also be analyzed for functionally-specific markers. These studies will determine whether HGF receptor/Met is activated and what HGF-dependent functions are stimulated in nonmalignant and malignant epithelial cells purified from human tissues and epithelial cell lines.

Second, we will determine if up-regulating or down-regulating HGF mRNA and protein expression in nonmalignant and malignant mammary epithelial cells affects the transformed and tumorigenic phenotypes of these cells in vitro and in vivo. On the one hand, conversion from paracrine to autocrine HGF stimulation, leading to sustained high level of HGF receptor/Met activation in breast epithelial cells, may be an important early step in breast neoplasia. On the other hand, disruption of an existing autocrine loop in mammary carcinoma cells may reduce their tumorigenic and metastatic potential. To test this possibility we are developing strategies to up-regulate (by gene transfection (58)) or down-regulate (by anti-sense oligonucleotides (59)) HGF mRNA expression in epithelial or carcinoma cells.

These studies provide new information about the role of PDGF and HGF in breast cancer progression. We are particularly interested in HGF as a possible target for pharmacological intervention in the treatment of breast cancer metastasis. Information from our work will be useful in the design of possible strategies involving antisense oligonucleotides to or proteinase inhibitors to block HGF expression and post-translational processing in mammary carcinoma cells, and possibly to suppress the metastatic progression of breast cancer.

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**LOCALIZATION OF PLATELET-DERIVED GROWTH FACTOR β -RECEPTOR
EXPRESSION IN THE PERI-EPITHELIAL STROMA OF HUMAN BREAST
CARCINOMA***

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*This work was supported by the National Cancer Institute of Canada and the United States Army Medical Research and Materiel Command grant No. DAMD 17-94-J-4407.

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Running title: PDGF β -receptors in human breast cancer

Key words: PDGF β -receptors/breast carcinoma/stromal cells

Footnote:

¹List of abbreviations: DCIS, ductal carcinoma *in situ*; HGF, hepatocyte growth factor; IDC, infiltrating ductal carcinoma; ISH, *in situ* hybridization; PDGF, platelet-derived growth factor; α -SMA, α -smooth muscle actin.

ABSTRACT

Platelet-derived growth factor (PDGF) BB is secreted by most human breast carcinoma cells, however only recently have PDGF β -receptors been demonstrated in malignant breast tissue. In the present study, the tissue localization of PDGF β -receptor expression was studied in human breast carcinoma and non-malignant breast tissues stained with both immunofluorescence and immunoperoxidase techniques. We examined a total of 29 cases of breast carcinomas, which showed both *in situ* and invasive components. PDGF β -receptor staining was localized in the peri-epithelial stroma, and was particularly intense in regions immediately adjacent to carcinoma *in situ* components in all tumors examined. A diffuse low level of PDGF β -receptor staining was seen throughout the stroma of 8 non-malignant breast tissues as well as non-malignant regions of tumor tissues. Image analysis was used to assess the coincidence of staining of PDGF β -receptor with epithelial or stromal cells in 13 of the 29 tumor tissues studied. Less than 5% of malignant ductal epithelium or myoepithelium showed PDGF β -receptor staining. Analysis with stromal cell type-specific markers indicated significant localization of PDGF β -receptor primarily with α -smooth muscle actin staining cells (32%) and vascular endothelial cells (41%) in the peri-epithelial stroma. PDGF β -receptor positivity was strongly associated with basement membrane surrounding regions of carcinoma *in situ*, but was less intense in regions of invasive carcinoma where basement membrane was degraded. The absence of PDGF β -receptors on carcinoma cells and their presence in the surrounding stroma suggests a paracrine stimulation of adjacent stromal tissue by malignant epithelial cells in human breast tumors.

INTRODUCTION

Growth factors which act in a paracrine or autocrine manner are important regulators of stromal-tumor interactions in breast cancer. Stromal fibroblasts and adipocytes secrete growth factors, such as keratinocyte-derived growth factor (1,2) and hepatocyte growth factor (HGF¹) (3), which can stimulate in a paracrine manner growth and invasiveness of mammary carcinoma cells. In addition, stimulation of stromal cells by carcinoma cells has recently been shown to induce expression of stromal proteinases (4,5), extracellular matrix proteins (e.g., tenascin (6) and thrombospondin (7)) and certain growth factors (e.g. HGF (8)). However the tumor-derived stimulatory components involved are not well understood.

PDGF BB, which is upregulated in the majority of breast cancer tissues (9-11) and in breast carcinoma cell lines (12-14), is an important candidate molecule in the paracrine stimulation of the stroma by carcinoma cells. PDGF BB has been shown to stimulate a variety of functions including growth (15), chemotaxis (16), and fibronectin matrix assembly (17) in connective tissue cells. PDGF is composed of two chains, A and B, held together by disulphide bonds and present in three dimeric forms AA, AB and BB (18,19). The dimers bind to two separate receptor types: α and β (18,19). The α -receptors bind to all three isoforms of PDGF, while the β -receptors have a very high affinity for PDGF BB, bind PDGF AB to some extent, but do not bind PDGF AA at all. The β -receptor has a ligand-activated protein kinase (20); the receptor is autophosphorylated in response to the appropriate ligand. Each PDGF chain is encoded by a distinct gene. The gene encoding PDGF B has been identified as the normal mammalian analogue of the *v-sis* oncogene (19,21,22). Fibroblast cells transfected with the PDGF B gene showed a

higher transforming activity than cells transfected with the PDGF A gene (23). The PDGF β -receptor may therefore represent an important regulatory pathway in breast cancer.

PDGF β -receptor mRNA and protein have been found on most stromal cell types including fibroblasts (24), smooth muscle cells (16), endothelial cells (25), and pericytes (26). Recently, some breast carcinoma cell lines (27), as well as breast cancer tissues (11,28,29) have also been shown to express PDGF β -receptors. In breast cancer tissues, PDGF β -receptors were expressed primarily in the stroma of normal and malignant breast tissue, not on non-malignant epithelial cells and only on a few malignant epithelial cells (11). However, it is not known what stromal cell types in breast cancer express PDGF β -receptors.

In the present study, we examined the presence of PDGF β -receptors in human malignant and non-malignant breast tissues, and used double immunofluorescence and *in situ* hybridization (ISH) techniques to localize their expression in specific cell types. Double immunofluorescence was studied by image analysis to quantify the degree of overlap of PDGF β -receptor stained areas with areas stained with antibodies specific for cell types. The results showed greater expression of PDGF β -receptor in the peri-epithelial stroma of regions of ductal carcinoma *in situ* (DCIS) than in stroma remote from epithelial cells. We therefore used specific markers for epithelium (cytokeratin), myofibroblasts and myoepithelial cells (α -smooth muscle actin [α -SMA] [30]), and endothelial cells (PAL-E) (31) to determine what cell types were involved. This study provides novel information about tissue localization of PDGF β -receptor in breast cancer tissue, and about possible cell targets for stimulation by PDGF BB.

MATERIAL & METHODS

Tissues: Tissue from surgical breast biopsies from patients with suspected breast cancer were snap frozen in liquid nitrogen within 20 minutes after acquisition and stored at -70°C . A total of 27 infiltrating ductal carcinomas (IDC), all of which had both regions of DCIS and invasive components, 1 infiltrating lobular carcinoma with lymph node involvement, and 1 DCIS were analyzed. Of the 27 IDCs examined, 13 showed positive lymph node involvement. Non-malignant breast tissues were obtained from the grossly normal regions surrounding the tumors and from 8 non-malignant breast lesions. Cryostat sections ($6\text{ }\mu\text{m}$) were fixed with cold (4°C) chloroform-acetone (50-50) for 5 minutes and stored at -70°C for future staining.

Immunofluorescence and immunoperoxidase staining: To block endogenous biotin in mammary tissue sections, an avidin-biotin blocking kit was used. Nonspecific binding sites were blocked with normal horse serum (1:5 dilution). For detection of PDGF β -receptors, tissue sections were incubated with mouse monoclonal anti-PDGF β -receptor antibody PDGFR-B2 (32) (kindly provided by K. Rubin, Uppsala, Sweden) at $1\text{ }\mu\text{g/ml}$ for 1 h, followed by biotinylated horse anti-mouse IgG (1:200 dilution) (Vector Labs) and Texas red-conjugated avidin (1:100) or horse radish peroxidase-conjugated avidin (Vector Labs). For immunoperoxidase staining, the immunoreaction was detected using a Vector Labs staining kit.

For staining cell markers or collagen Type IV, nonspecific sites for the second antibody were blocked with normal goat serum (1:5 dilution). The second antibodies used were: (1) Mouse monoclonal antibody (CAM 5.2) against human cytokeratins 8 and 18 (Becton Dickinson),

used at 25 $\mu\text{g/ml}$ to stain epithelium; (2) monoclonal anti- α -smooth muscle actin (α -SMA) antibody 1A4 (Sigma Immunochemicals), used at 1:400 dilution to stain smooth muscle-related components such as myoepithelium and myofibroblasts (30); (3) monoclonal antibody PAL-E (Monosan), used at 1:12 dilution to stain non-arterial endothelial cells (31) (The endothelial nature of the PAL-E positive cells was confirmed by positive staining for Factor VIII [data not shown].); (4) polyclonal rabbit anti-common acute lymphoblastic leukemia antigen (CALLA) antiserum A12 (33) (kindly provided by S. Carrel, Bern, Switzerland), used at 1:25 dilution to stain myoepithelial cells, and (5) goat anti-collagen Type IV IgG used at 25 $\mu\text{g/ml}$ (Southern Biotechnologies Associated). All second antibodies were incubated with tissues for 30 minutes; binding of second antibodies was detected by affinity-purified FITC-labelled goat anti-mouse or anti-rabbit IgG used at 1:100 dilution. All washes were done with PBS. Normal mouse IgG (1 $\mu\text{g/ml}$) without primary antibodies was used as a control. Slides were mounted with Fluoromount-G (Southern Biotechnologies Associated) and were examined under a Leica fluorescence microscope equipped with an Hb 100 light source. Each field was viewed with an I3 filter for FITC illumination and an N2.1 filter for Texas red illumination. Hematoxylin and eosin staining of the frozen sections was performed to determine tissue morphology.

Image Analysis: Microscope images of identical fields were photographed under illumination for Texas red or FITC with TMAX 400 film. Black and white negatives of the FITC and Texas red fluorescence were analyzed using an M2 Image Analysis Software programme (Image Analysis Systems, Brock University, Ont., Canada). To compare staining of PDGF β -receptor in different regions, the ratio of mean fluorescence intensity of total positive target to that of the peri-epithelial

stroma or of the remote stroma (i.e. distant from epithelium) was calculated. (See Table 1).

To determine the proportion of tissue area stained with both PDGF β -receptor and a specific cell marker, the total number of pixels positive for both FITC and for Texas red fluorescence in an identical field was calculated. The overlap of FITC with Texas red was then calculated when the two regions were superimposed. The proportion of pixels corresponding to double staining was expressed as the per cent of double-positive (FITC + Texas red) pixels divided by the total FITC-positive pixels. (See Table 2.)

***In situ* hybridization (ISH):** ISH was performed by a modification of the procedure of Yang and Park (34). Tissues were fixed in 4% paraformaldehyde, processed in alcohol, and embedded in paraffin. Sections (6 μ m) were dewaxed in toluene, and rehydrated. Permeabilization was performed by treating with 0.2 M HCl, 0.2% Triton X-100 in PBS, and 40 μ g/ml proteinase K. Slides were then washed in 0.1 X PBS, refixed in 4% paraformaldehyde, washed again in 0.1 X PBS and acetylated using 0.25% acetic anhydride in 0.1M triethanolamine HCl. Slides were then dehydrated, delipidated in 100% chloroform followed by absolute ethanol and 95% ethanol, and then air-dried.

Digoxigenin-labelled RNA riboprobes were synthesized from a plasmid containing a 1-kb Pst I fragment of cDNA corresponding to the external domain of PDGF β -receptor (34). The probe was diluted in prehybridization mix at 15 ng/ μ l, and hybridization mix (200 μ l) was applied to each section. Slides were incubated at 42°C overnight, followed by washing in 1X SSC, rinsing in RNase buffer (0.5 M NaCl, 10 mM PIPES [pH 7.2], 0.1% Tween 20), and digesting unbound single-stranded RNA by incubation in 20 μ g/ml RNase A (Sigma). Slides were then

washed in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and blocked with 3% normal sheep serum in buffer 1. To detect specific hybrids, slides were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) (1:1000), then washed twice with buffer 1, and twice in buffer 2 (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl_2). Hybrids bound to anti-digoxigenin antibody were then visualized by a colour reaction containing nitroblue tetrazolium salt (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and Levamisole (0.24 mg/ml) in buffer 2 (kit from Vector Laboratories, Burlingame, CA). Slides were then dehydrated, washed in xylene, mounted with permount, and viewed and photographed under bright field illumination with a Leica microscope. RNA specificity of probe binding was tested by treating control slides with 20 $\mu\text{g/ml}$ RNase A in RNase buffer at 37°C prior to hybridization. Specificity of binding was also verified by the pattern of expression in tissues known to express PDGF β -receptor (e.g. skin, placenta (35)), and by comparison with tissue-compartment-specific expression of other positive control probes (e.g. HGF receptor [Met] expression [A. Tuck, M. Park, E. Sterns, A. Boag and B. Elliott, manuscript submitted]).

RESULTS

PDGF β -receptor expressing cells are localized in the peri-epithelial-stroma in regions of mammary ductal carcinoma *in situ*: Our initial approach was to compare the tissue localization of PDGF β -receptor protein in 29 breast tumor tissues with that in non-malignant breast tissues. Immunofluorescence staining revealed intense PDGF β -receptor protein expression localized in the peri-epithelial stroma, particularly in regions of DCIS (Figure 1a-1c). A similar pattern of staining was observed when tissues were stained using an immunoperoxidase technique (data not shown). ISH analysis showed that cells in the peri-epithelial stroma and adjacent capillary endothelium express the corresponding PDGF β -receptor mRNA (Figure 2). In contrast to malignant breast, regions of normal breast tissue showed a less intense, more diffuse staining pattern of PDGF β -receptors in the stromal region (Figure 1d-1f).

To quantify the relative amount of PDGF β -receptor protein staining in the peri-epithelial stroma and in the stroma remote from epithelial cells, we carried out image analysis of malignant and non-malignant tissue sections stained with anti-PDGF β -receptor antibody (Table 1). The results showed that most PDGF β -receptor staining of malignant tissue was localized in the peri-epithelial stroma. The mean fluorescence intensity of PDGF β -receptor staining of the remote stroma was significantly less than that at the peri-epithelial stroma. In non-malignant regions, both the peri-epithelial and the remote stroma showed a mean fluorescence intensity which was significantly less than that of the mean intensity of the PDGF β -receptor-positive target area. Thus, PDGF β -receptor expression was localized to the peri-epithelial stroma in regions of DCIS, and was more diffuse throughout the stroma in non-malignant breast tissue.

PDGF β -receptor is expressed by α -smooth muscle actin (α -SMA) positive cells and endothelial cells in the peri-epithelial stroma of breast cancer tissues: To identify the stromal cell type(s) expressing PDGF β -receptors, we carried out a double staining procedure with anti-PDGF β -receptor antibody and antibodies against cytokeratins 8 and 18 (epithelial cells), α -SMA (myoepithelium, myofibroblasts, or pericytes), or PAL-E (non-arterial endothelial cells) (Figure 1, 3 and 4). Image analysis revealed $< 5\%$ overlap of the cytokeratin positive cells with PDGF β -receptor staining regions (Table 2). In contrast, an average of 32% of the α -SMA-staining areas in all tumors examined showed PDGF β -receptor positivity, particularly surrounding regions of DCIS. In addition, strong co-localization (averaging 41%) of PAL-E-staining areas with PDGF β -receptor staining areas was also observed in both regions of DCIS and invasive carcinoma. No significant coincidence of PDGF β -receptor staining with CALLA-expressing myoepithelial cells was detected (Table 2). We conclude from these studies that PDGF β -receptor is present in smooth-muscle-related cells (myofibroblasts or pericytes) and non-arterial endothelial cells in the peri-epithelial stroma of malignant breast tissue.

PDGF β -receptor expressing cells are associated with basement membrane: To further assess the anatomical location of PDGF β -receptor staining cells within the gland, we determined their position with respect to basement membrane. Double immunofluorescence staining for PDGF β -receptor and collagen Type IV, a prominent basement membrane protein, was therefore carried out. The results showed close association of collagen Type IV and PDGF β -receptor staining in

the peri-epithelial regions of DCIS in all tumors examined (Figure 6a-6c). These results indicate that PDGF β -receptor positive cells are associated with basement membrane structures. Basement membrane of invasive regions became fragmented and disorganized (Figure 6d). In these invasive regions, PDGF β -receptor expression was detected primarily in cells associated with remaining basement membrane fragments, but was decreased or absent in regions where basement membrane was degraded.

DISCUSSION

PDGF BB is secreted by many breast cancer cell lines and has recently been shown to be up-regulated in breast cancer (9-11). However, only recently have PDGF β -receptors been demonstrated in malignant tissue (11). In the present report, we showed that PDGF β -receptor protein is essentially absent in both non-malignant and malignant breast epithelial cells. However, PDGF β -receptor expression was intense and localized in the peri-epithelial stroma in regions of DCIS; whereas, regions of non-malignant stroma displayed a more diffuse, weaker expression of PDGF β -receptors. These findings coincide with the recent report by Coltrera *et al.* (11) that PDGF β -receptor is expressed in fibroblastic stromal cells in non-malignant breast and DCIS. We have further demonstrated that the intense PDGF β -receptor staining in malignant breast tissue was co-localized with α -SMA staining cells and endothelial cells near intact basement membrane surrounding malignant ducts and capillaries, respectively, but not with myoepithelial cells. In regions of invasive carcinoma, PDGF β -receptor expression was less intense where basement membrane was degraded. These results imply a paracrine stimulation by malignant epithelium of specific cell types in adjacent stroma.

PDGF β -receptor-expressing α -SMA-positive cells could be myoepithelium, myofibroblasts or vascular smooth muscle cells (30). Double staining for PDGF β -receptor and the myoepithelial cell marker, CALLA (32), showed that myoepithelial cells, though adjacent to PDGF β -receptor-positive areas, did not themselves express PDGF β -receptor. Recently, Ronnov-Jessen *et al.* (36) have shown that malignant breast epithelium can stimulate differentiation of α -SMA-expressing myofibroblasts from fibroblasts, concomitant with migration

of myofibroblasts toward the carcinoma cells in a serum-free 3-dimensional "tumor environment" culture system with purified cell types. Thus myofibroblasts may be attracted to the vicinity of the basement membrane by malignant epithelial cells which produce PDGF BB. PDGF BB could be involved either directly by stimulating chemotaxis (15,37), or indirectly by stimulating production of other growth factors (e.g. transforming growth factor- β (17), basic fibroblast growth factor (38), and insulin-like growth factor-I (39,40)). Thus, stimulation of fibroblast proliferation and chemotaxis by PDGF BB may be at least partially responsible for the pronounced desmoplastic response in breast cancer.

Strong co-localization of PDGF β -receptors with endothelial cells (PAL-E positive) was observed. Many of the PDGF β -receptor-expressing endothelial cells are associated with capillary-like structures adjacent to regions of DCIS and in invasive carcinoma, as identified by Factor VIII staining (data not shown). These findings are consistent with the observation that PDGF BB, which binds to PDGF β -receptors, can stimulate angiogenesis *in vitro* and *in vivo* (41,42). Although the mechanism by which PDGF BB stimulates angiogenesis *in vivo* is not known, recent reports suggest that thrombospondin, which is secreted in response to PDGF (43), may be involved (44). In support of this hypothesis, Clezardin *et al.* (7) have shown that thrombospondin and its receptors CD36 and CD51 are upregulated in hyperplastic and neoplastic human breast tissue. We have also demonstrated increased expression of thrombospondin in the peri-epithelial regions of DCIS and invasive carcinoma (data not shown). PDGF BB may therefore exert its angiogenic effect by stimulating thrombospondin production.

PDGF β -receptor positive cells were found to be closely localized to basement membrane regions of DCIS. In invasive regions, PDGF β -receptor expression remained associated with cells

adjacent to fragmented basement membrane or vascular regions; however, where no basement membrane was apparent, expression of PDGF β -receptor was less intense. We also observed occasional expression of PDGF β -receptor mRNA in malignant epithelium (data not shown), similar to recent results reported by Caltera *et al.* (11). However PDGF β -receptor protein was not detected on malignant or non-malignant epithelium in our study. This observation may reflect the fact that our staining procedure was carried out on unfixed frozen sections and would therefore detect primarily membranous receptors. Together, our results suggest that PDGF BB acts during early stages of breast cancer, e.g. at the level of DCIS, on adjacent PDGF β -receptor-expressing myofibroblasts and endothelial cells. Possible effects of PDGF BB on these two cell types could be increased proliferation (15) and angiogenesis (41).

Recently, basal cell carcinoma (45) and colorectal carcinoma (46) have been independently shown to express PDGF AB/BB, and the corresponding receptors can be detected on adjacent stromal components. Ponten *et al.* (45) observed expression of PDGF α - and β -receptors in the specific stromal components surrounding basal cell carcinoma. The A and B chains of PDGF were found mainly in the basal cell carcinoma cells, in hair matrix and in sweat gland epithelium. Likewise, Sundberg *et al.* (46) have demonstrated that microvascular pericytes express PDGF β -receptors in human healing wounds and in colorectal adenocarcinomas. PDGF β -receptors and PDGF B chain-expressing cells were found to be in close proximity, suggesting a role for PDGF in the physiology of the microvasculature in these lesions. PDGF has also been shown to be directly responsible for induction of vascular connective tissue stroma by xenotransplanted human melanoma cells which produce PDGF BB (42). Our results provide strong support for a similar mechanism for stimulation of the stroma by adjacent carcinoma cells.

In summary, our findings are consistent with a paracrine mechanism in which PDGF β -receptor is upregulated in stromal cells immediately adjacent to tumor cells which have been shown previously to produce PDGF BB (11). This upregulation of PDGF β -receptors was found to be localized with endothelial cells and α -SMA-positive cells in the peri-epithelial region of breast carcinomas. These results suggest a role of the PDGF β -receptor pathway in the desmoplastic and angiogenic response of adjacent stroma in breast cancer. Future studies will examine the possible functional role of PDGF β -receptor activation in epithelial-stromal interactions in breast cancer.

ACNOWLEDGEMENT: The authors wish to thank Dr. K. Rubin for his generous gift of PDGFR-B2 antibody. The excellent technical assistance of Marian Arnold and Lloyd Kennedy are greatly appreciated.

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TABLE 1**Relative PDGF β -receptor Expression in the Peri-epithelial Stroma and the Remote Stroma of Non-malignant and Malignant Breast Tissue**

<u>Tissue^a</u>	<u>N</u>	<u>Peri-epithelial Stroma^b</u>	<u>Remote Stroma^b</u>
Non-malignant	14	0.73 ± 0.08	0.71 ± 0.07
Malignant	26	$1.00^* \pm 0.01$	0.70 ± 0.08

Legend:

- a) Tissue sections were stained with monoclonal mouse anti-PDGF β -receptor antibody and biotinylated goat anti-mouse IgG detected with rhodamine-conjugated avidin as described in Figure 1. Tissues were viewed under epifluorescence illumination and photographed with T-MAX Kodak film. N indicates the number of tissue sections per group analyzed. A total of 5 non-malignant lesions and 6 tumors with regions of DCIS was analyzed.
- b) Image analysis was carried out to determine the ratio of mean intensity of total PDGF β -receptor staining to that of the peri-epithelial stroma or of the remote stroma (i.e. distant from epithelium). The asterisk indicates significantly greater intensity of PDGF β -receptor staining in the peri-epithelial stroma than in the remote stroma in malignant breast tissue ($p < 0.05$ as determined by analysis of variance). A total of 25 comparisons per tissue section were carried out. Results are expressed as mean \pm S.D.

TABLE 2**Localization of PDGF β -receptors with various Cell Markers in Human Breast Carcinomas**Percentage of cell marker-positive pixels coincident with PDGF β -receptor^b:

<u>Cytokeratin 8 & 18</u> <u>(epithelium)</u>	<u>CALLA</u> <u>(myoepithelium)</u>	<u>α-SMA</u> <u>(smooth muscle-related)</u>	<u>PAL-E</u> <u>(endothelium)</u>
2.6 \pm 4.4 (9)	4.9 \pm 3.8 (7)	31.9 \pm 25.2 (10)	41.0 \pm 20.2 (13)

Legend:

- a) Frozen sections (6 μ m) of malignant human breast biopsies were stained by double immunofluorescence with anti-PDGF β -receptor antibody and antibody against either cytokeratins 8 and 18 (epithelial cells), CALLA (myoepithelial cells), α -SMA (smooth muscle-related cells), or PAL-E (non-arterial endothelial cells). See Materials and Methods for antibody details.
- b) Slides were photographed under epifluorescence illumination for Texas red (PDGF β -receptor) or FITC (cell markers). The total area in pixels of Texas red and FITC positivity in each section was independently determined using an image analysis system. The percentage of double-positive pixels divided by the total FITC-positive pixels is shown. Values represent the mean \pm S.D. The number of tumors analyzed for each cell marker is indicated in parentheses.

FIGURE LEGENDS:

Figure 1: Localization of cytokeratins 8 and 18, and PDGF β -receptor expression in non-malignant tissue and DCIS. Sections ($6\ \mu$) from malignant and non-malignant breast tissue were stained using (a,d) hematoxylin and eosin to identify tissue morphology. Adjacent sections were also stained using double immunofluorescence as follows: (b,e) with monoclonal anti-cytokeratin 8 and 18 antibody detected by FITC-conjugated anti-mouse IgG to identify epithelial cells, and (c,f) with monoclonal anti-PDGF β -receptor antibody detected by biotinylated anti-mouse IgG and Texas red-avidin, as described in Materials and Methods. The same fields were viewed under FITC illumination and Texas red illumination. Panels a-c show a region of DCIS. Panels d-f show a non-malignant breast lobule.

Figure 2: ISH analysis of PDGF β -receptor expression in DCIS. Breast tissue with DCIS was stained using ISH for PDGF β -receptor as described in Materials and Methods. The results showed positivity at the peri-epithelial stroma (arrow). Negative controls included sections treated with RNase A and hybridized with vector-only riboprobes (not shown). T, malignant epithelium; C, capillary endothelium.

Figure 3: Localization of α -smooth muscle actin and PDGF β -receptor expression in DCIS. A breast tumor tissue section showing DCIS was stained using (a) hematoxylin and eosin to identify tissue morphology. An adjacent section was also stained using double-immunofluorescence with (b) monoclonal anti- α -SMA which identifies myofibroblasts,

myoepithelial cells, and pericytes, and (c) monoclonal anti-PDGF β -receptor antibody, as described in Figure 1.

Figure 4. Localization of PAL-E (endothelial) marker and PDGF β -receptor expression in DCIS. A breast tumor tissue section showing DCIS was stained using (a) hematoxylin and eosin to identify tissue morphology. An adjacent section was also stained using double-immunofluorescence with (b) monoclonal antibody PAL-E which identifies non-arterial endothelial cells, and (c) monoclonal anti-PDGF β -receptor antibody, as described in Figure 1.

Figure 5. Localization of collagen Type IV and PDGF β -receptor expression in DCIS and in invasive carcinoma. Breast tumor tissue sections showing DCIS and invasive carcinoma were stained using (a,d) hematoxylin and eosin to identify tissue morphology. Adjacent sections were also stained using double immunofluorescence as follows: (b,e) with anti-collagen Type IV IgG which identifies basement membrane, and (c,f) with monoclonal anti-PDGF β -receptor antibody, as described in Figure 1. Panels a-c show a region of DCIS; panels d-f show a region of invasive carcinoma.

Figure 1

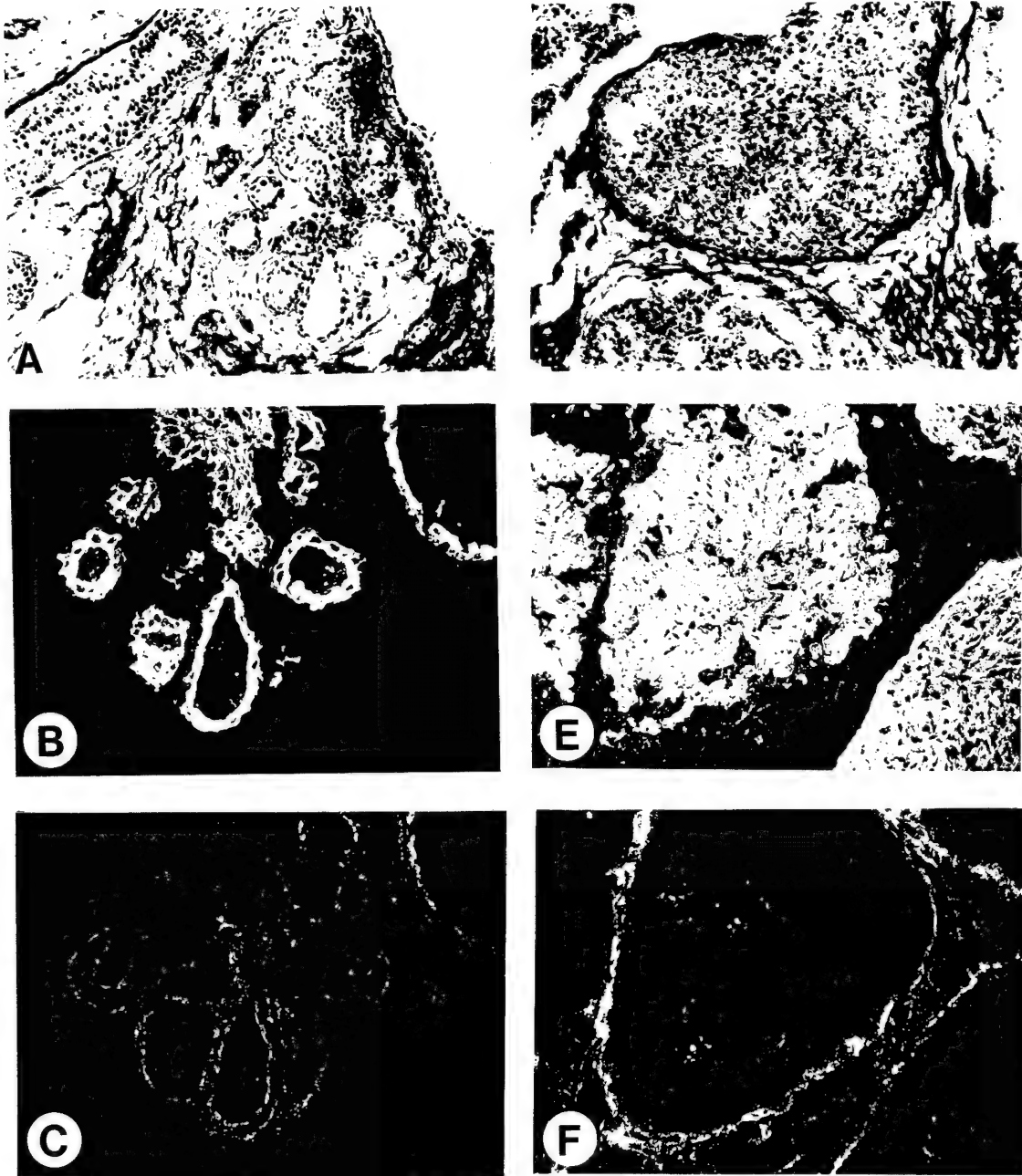


Figure 2



Figure 3

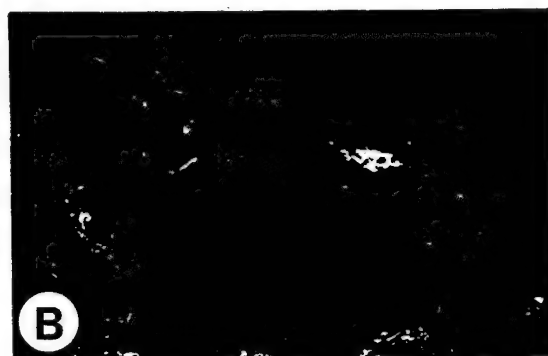
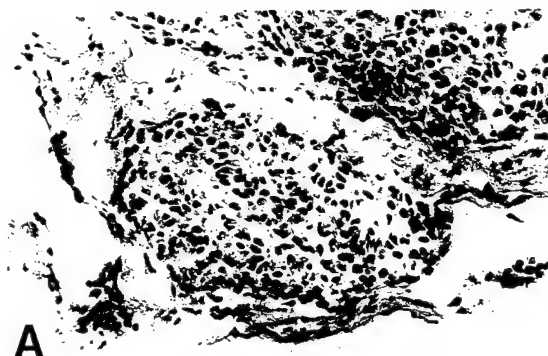


Figure 4

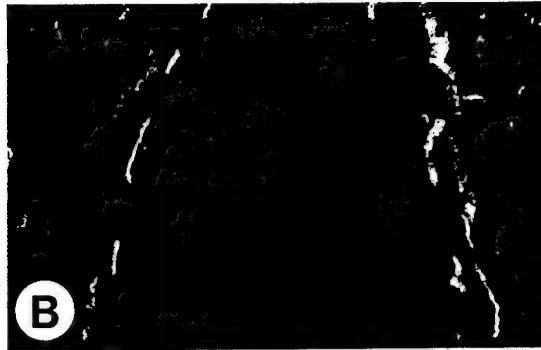
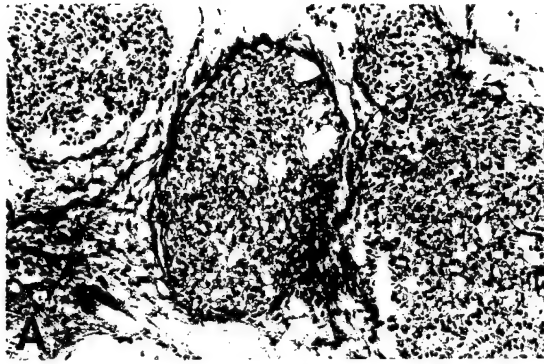
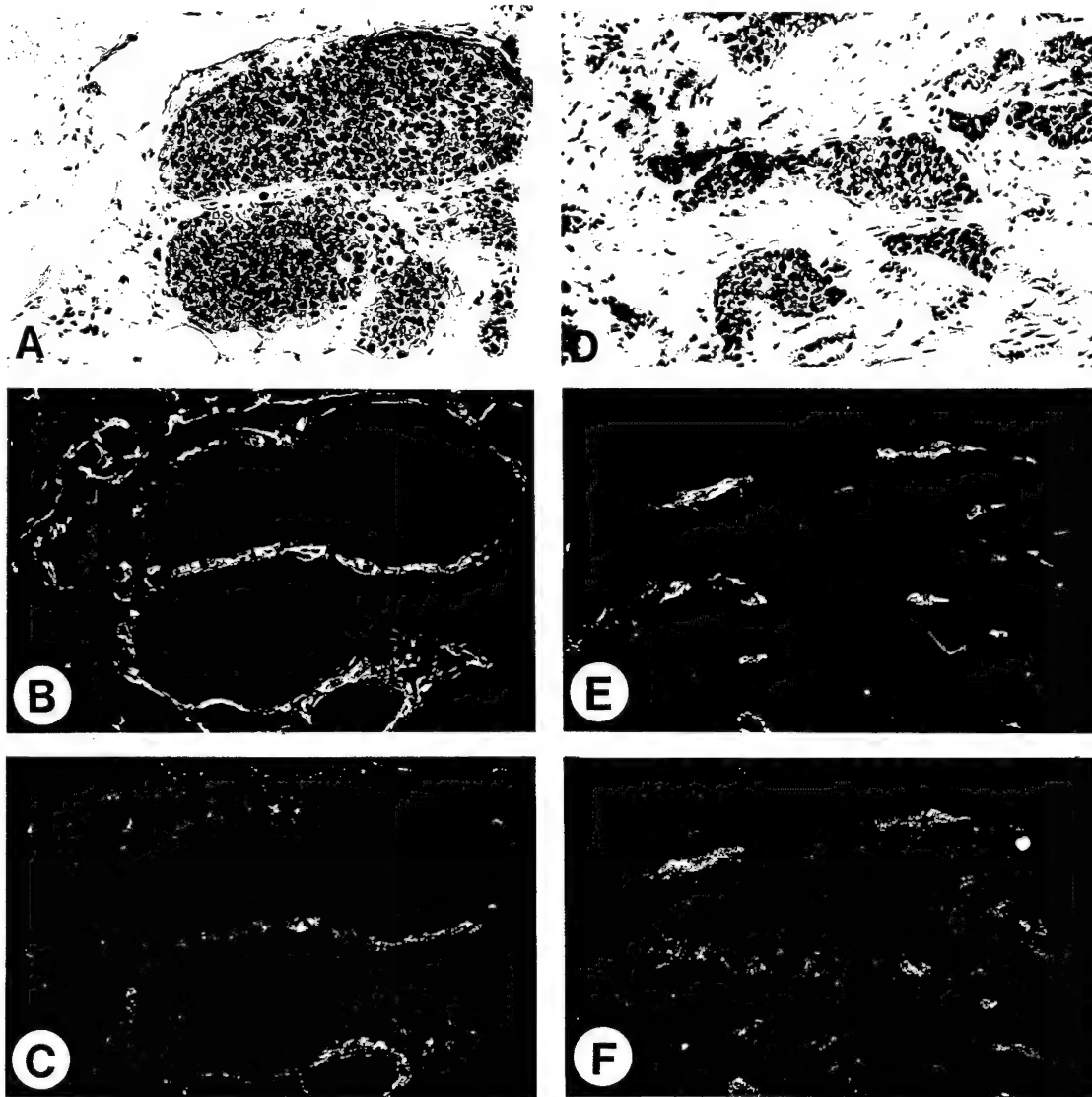


Figure 5



**CO-EXPRESSION OF HEPATOCYTE GROWTH FACTOR (HGF)
AND RECEPTOR (MET) IN HUMAN BREAST CARCINOMA**

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Supported by grants from the National Cancer Institute of Canada (No. 3083), the U.S. Army Breast Cancer Research Initiative (DAMD 17-94-J-4407) and the Clare Nelson Bequest Research Fund, Kingston General Hospital.

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Running title: HGF and Met in breast cancer

Text pages: 24 Figures: 3

ABSTRACT

Expression of hepatocyte growth factor (HGF) and HGF receptor (HGFR, product of the *met* proto-oncogene) mRNA were examined using non-isotopic *in situ* hybridization (ISH) in a spectrum of benign and malignant human breast tissues. mRNA for both HGFR and HGF was detected in benign ductal epithelium. Epithelial expression of HGF mRNA was particularly intense in regions of ductal epithelial hyperplasia. Positive expression of HGF (but not HGFR) mRNA was also found in adipocytes, endothelial cells, and to varying degrees in stromal fibroblasts. In 12 of 12 cases of ductal carcinoma *in situ* and infiltrating ductal carcinoma, carcinoma cells showed a heterogeneous pattern of expression for both HGFR and HGF mRNA. In infiltrating ductal carcinomas, intense expression of HGFR mRNA was not restricted to ductular structures, but was also seen in non-duct-forming carcinoma cells. The same zones of the tumors (most commonly at the advancing margins) that expressed strongly HGFR mRNA often were also strongly positive for HGF mRNA, suggesting a possible autocrine effect. The expression pattern of HGFR protein in 25 cases including the same series of tissues used for ISH analysis was similar to that of HGFR mRNA, as determined by an immunoperoxidase technique. The finding that HGFR is expressed by both benign and malignant epithelium, and is not restricted to duct-forming structures, suggests that although the potential for HGF/HGFR binding is maintained in malignancy, the response to ligand binding at the level of the receptor, or the cellular response to receptor activation may change at some point during progression.

INTRODUCTION

Hepatocyte growth factor receptor (HGFR), also known as the product of the *met* proto-oncogene, is expressed by a variety of epithelial (and a few non-epithelial) cell types, including mammary epithelium (1-4). The ligand, hepatocyte growth factor (HGF) was originally described as a mitogen for hepatocytes (5) and later found to be identical to Scatter Factor (SF), a modulator of intercellular organization (6). HGF has been found to be produced and secreted by cells of the mesenchymal/stromal compartment (7-12), although recent evidence has shown epithelial expression of HGF in certain instances (13-16). HGF has been shown to control a variety of cell functions including cell growth (10,17,18), movement (19-22), invasiveness (21,22) and cell-cell adhesion (23,24), as well as morphogenesis of epithelial (eg. ductular, tubular) structures (2,25-27). Many of these functions have been shown to require activation of the Met receptor (28, 29).

There is presently controversy over the possible role of HGFR in human breast cancer. Both the HGF (6) and HGFR (30) genes have been mapped to human chromosome 7q. Loss of heterozygosity (LOH) at 7q has been found to be a frequent event in human breast cancer (40% of informative cases), and LOH at 7q in human breast tumors has been found to correlate with shorter "metastasis-free" and overall survival times (31). In addition, Tsarfaty *et al.* (2) have reported that HGFR protein is expressed in benign ductal epithelium, and is expressed at lower levels in the immediately adjacent malignant ductal epithelium. Although collectively these results

are consistent with a possible "tumor suppressor" role for HGF and/or HGFR in human breast carcinoma, this hypothesis has yet to be proven. Other studies have shown HGF to act as a motogen or morphogen in most breast carcinoma cell lines tested (2,22); and there are a few reports of HGF acting as a mitogen for mammary epithelial cells (10,18). Wang *et al.* (16) reported that HGF mRNA is expressed in both benign and malignant mammary epithelium, and that the most abundant expression in benign epithelium was in regions of proliferative activity. Although they suggested a possible autocrine role for HGF in inducing proliferation of benign and malignant mammary ductal epithelium, they had not examined expression of HGFR mRNA or protein in these same tissues to support this contention. Finally, the clinical import of HGF in human breast carcinoma was highlighted recently in the work of Yamashita *et al.* (32), who reported that a high level of expression of HGF protein is an even more significant factor in predicting poorer relapse-free and overall survival than is lymph node status. However, this study did not examine the stromal or epithelial source of the HGF in breast cancer tissues.

We thus regarded it to be important to establish more clearly the cellular expression pattern of both HGF and HGFR in benign and malignant human breast tissues. We examined expression of both genes at the mRNA level in serial sections of the same tissues, using *in situ* hybridization (ISH).

MATERIALS AND METHODS

Tissues

Mastectomy and segmentectomy/lumpectomy specimens were obtained fresh immediately following excision. Sections of both benign and malignant breast taken for ISH were fixed in 4% paraformaldehyde in PBS for 4 hours, followed by alcohol cycle processing and embedding in paraffin. Sections of normal skin were taken for use as controls for HGFR expression from associated (usually nipple region) skin. Placental tissues as controls for HGF expression were obtained fresh from normal term deliveries, and also fixed immediately in 4% paraformaldehyde for 4 hours. For ISH, tumor specimens included 2 cases of ductal carcinoma *in situ* (DCIS) alone, 2 cases of well-differentiated, 5 cases of moderately differentiated and 3 cases of poorly differentiated infiltrating ductal carcinoma (IDC). For immunohistochemistry, an additional 13 specimens, including 1 well-differentiated, 7 moderately differentiated, and 5 poorly differentiated IDC were analyzed. Access to tissues satisfied the requirements of the Kingston General Hospital ethics committee.

Plasmids

Both the HGF and HGFR (*met*) plasmids used for generation of riboprobes consisted of a cDNA fragment of the gene of interest cloned into a Bluescript KSII⁺ vector between the T3 and T7 promoters, each with the 5' end of the cDNA downstream to the T7 promoter. The HGF cassette consisted of the 540 bp BamHI-XhoI fragment of the human HGF cDNA (33). The HGFR cassette consisted of the

800 bp EcoRI-EcoRV fragment of the human *met* cDNA (34). The platelet-derived growth factor (PDGF) α -receptor plasmid, used for generation of control riboprobe, consisted of the 1.5 kb EcoRI-PstI fragment (extracellular domain) of the cDNA cloned into pGEM-Blue between the T7 and SP6 promoters, such that the 5' end lay downstream of the T7 promoter. The PDGF α -receptor plasmid was obtained as a kind gift from Dr. Lena Welsh and Dr. Keiko Funa (35).

Riboprobes

Riboprobes were generated by *in vitro* transcription from linearized templates with the appropriate phage RNA polymerase (Promega Corp., Madison, WI) in the presence of digoxigenin-UTP (Boehringer Mannheim, Montreal, PQ). Antisense riboprobes for HGF and HGFR were generated by transcription from the T3 promoter, whereas PDGF α -receptor antisense (control) riboprobes were generated by transcription from the SP6 promoter. Riboprobes generated from "vector-only" templates were also used as negative controls.

In Situ Hybridization

ISH was performed by a modification of the procedure of Yang and Park (36). Six-micron paraffin sections were cut onto baked slides coated with triethoxysilane (Sigma, St. Louis, MO), dewaxed in toluene, and rehydrated. Permeabilization was performed by treating at room temperature sequentially with 0.2M HCl, 0.2% Triton X-100 in PBS, and 40 μ g/ml proteinase K for 10 min each. Slides were then washed

in 0.1X PBS, refixed for 30 min at room temperature in 4% paraformaldehyde, washed again in 0.1X PBS and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine HCl. Slides were then dehydrated, delipidated in 100% chloroform for 15 min followed by absolute ethanol for 5 min and 95% ethanol for 15 min, and then air-dried.

Probes were diluted in prehybridization mix at 15 ng/ μ l, and 200 μ l of hybridization mix was applied to each section. Slides were incubated in a humid chamber at 42°C overnight, washed in 1X SSC at 55°C for 30 min, rinsed in RNase buffer (0.5 M NaCl, 10 mM PIPES [pH 7.2], 0.1% Tween 20) at room temperature for 10 min, and incubated in 20 μ g/ml RNase A (Sigma) for 30 min at 37°C to remove unbound single-stranded RNA. Slides were washed in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) at room temperature for 10 min and blocked with 3% normal sheep serum in buffer 1 at room temperature for 30 min. To detect specific hybrids, slides were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) (1 to 1,000 dilution in buffer 1 with 3% normal sheep serum) for 30 min, then washed twice (10 min each) with buffer 1, and twice (5 min each) in buffer 2 (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl₂). Hybrids bound to anti-digoxigenin antibody were then visualized by a color reaction containing nitroblue tetrazolium salt (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and Levamisole (0.24 mg/ml) in buffer 2. (An alkaline phosphatase substrate kit IV [BCIP/NBT] [Vector Laboratories, Burlingame, CA] was used). Color was allowed to develop for 12 hours in the dark. Slides were then dehydrated, washed

in xylene, mounted with permount, and viewed and photographed by a light microscope. Positive areas showed brown-purple cytoplasmic staining (Figs. 1 & 2).

Testing for RNA specificity of probe binding was performed by (a) treating sets of control slides with 20 μ g/ml RNase A in RNase buffer for 30 min at 37°C prior to hybridization, and (b) hybridizing with vector-only riboprobes generated from the parent vector without the HGF or HGFR cassette, transcribed in the same direction as the antisense probes. Controls for cell specificity of binding included testing of the pattern of binding of antisense HGFR and HGF probes to known positive tissue targets (e.g. placenta [i.e., trophoblast] for HGFR mRNA, and skin [i.e., epidermis and adenexal epithelium] for HGF mRNA).

Immunohistochemistry

Paraffin-embedded sections of formalin-fixed breast tissues from 25 cases of DCIS and IDC including the same set of surgical pathologic specimens used for ISH analysis were assessed for HGFR expression by a immunoperoxidase technique. Samples were immunostained with a polyclonal anti-met antibody raised against a COOH-terminal peptide (1:200) (37), using a modification of the avidin-biotin peroxidase complex method previously described (38). The immuno-reaction was visualized using diaminobenzidine and H₂O₂. The antibody used has been shown previously to detect human Met (HGFR) specifically by both immunoprecipitation and Western blotting (15,37).

RESULTS

Both HGFR and HGF mRNA were expressed cytoplasmically in regions of non-malignant ductal epithelium in all tissues examined (Fig. 1). Expression of HGF mRNA in benign ductal epithelium was variable, but appeared most intense in regions showing architectural evidence of ductal epithelial hyperplasia. Expression of HGF (but not HGFR) mRNA was also found to varying degrees in adipocytes, endothelial cells and stromal fibroblasts (Fig. 1B). Ductal carcinoma *in situ* (DCIS) and infiltrating ductal carcinoma (IDC) cells in all tissues examined showed a heterogeneous pattern of expression of both HGFR and HGF mRNA (Fig. 2). In 11 of 11 IDC examined, intense expression of HGFR mRNA was not restricted to ductular structures, but was seen also in non-duct-forming carcinoma cells (Fig. 2D). There was no apparent difference in patterns of HGF mRNA and HGFR mRNA in IDC of different grades. The same zones of a tumor that were strongly positive for HGFR mRNA were commonly also strongly positive for HGF mRNA expression, suggesting a possible autocrine loop effect (eg. Fig. 2A and 2C). Where heterogeneity of expression for HGF and HGFR mRNA was apparent within a tumor, strongest positivity for both was usually seen at or near the advancing margins of the tumor (Fig. 2A and 2C), whereas expression in more central regions of the tumor was less intense (Fig. 2B). This was true for 9/10 tumors which included sampling of the advancing margin. This effect was seen most commonly in the absence of morphologic evidence of ischemia/necrosis in more

central regions of the tumor, such that higher level expression of mRNA for both HGF and HGFR at the advancing margins is unlikely due to ischemia effect alone.

Pretreatment of slides with RNase A eliminated all positive signals. Likewise, riboprobes generated from the parent vector lacking the HGF or HGFR insert showed no positivity (not shown). ISH of skin sections for HGFR mRNA showed the expected pattern of strong positivity in immature layers of the epidermis and in the adnexal epithelium (5,39), and expression of HGF mRNA in placental tissue showed the expected pattern of strong positivity in the trophoblast (40) (not shown). These controls verify that HGF and HGFR probes were in fact binding to the corresponding mRNA. Analysis of breast tissue with a control antisense PDGF α -receptor riboprobe showed the predicted pattern of weak positive expression in stromal fibroblasts, endothelial cells, and inflammatory cells, with no expression in benign or malignant ductal epithelial cells (not shown). Similarly, analysis of skin sections for PDGF α -receptor expression showed positivity in dermal fibroblasts and endothelial cells, with no expression in the epidermis, as reported previously by Pontén *et al.*, (41) (not shown). Collectively, these controls confirmed the specificity of the ISH procedure.

Immunoperoxidase analysis of HGFR protein expression showed a pattern of staining closely reflecting that seen for expression of the corresponding mRNA (Fig. 3A-D). In 25 of 25 cases examined, strong expression of HGFR protein was seen in both benign and malignant epithelium, and was not restricted to ductal or acinar structures. Most intense positive staining for HGFR protein was apparent at or near the advancing margins of invasive carcinomas (Fig. 3B).

DISCUSSION

The ISH data reported here show that HGF mRNA is expressed by benign and malignant mammary ductal epithelium, as well as variable expression by stromal cells (fibroblasts, adipocytes and endothelial cells), in a manner similar to that described by Wang et al. (16). Also in agreement with Wang *et al.* (16), we found intense expression of HGF mRNA in benign ductal epithelium in regions of ductal epithelial hyperplasia, as well as in malignant epithelium of different histologic grades.

Expression of HGFR mRNA was detected in both benign and malignant ductal epithelium, consistent with the demonstration by Tsarfaty *et al.* (2) of HGFR protein expression in similar tissues. However, in contrast to what has been described previously (2), we have found that expression of HGFR mRNA and protein levels are not decreased uniformly in malignant epithelium, and expression of HGFR is not limited to cells lining ductules or tubules. In fact, we have found that both *in situ* and invasive ductal carcinomas of all grades show a heterogenous pattern of HGFR expression. Individual malignant cells or groups of cells of poorly differentiated IDC were commonly found to stain strongly for both HGFR mRNA and protein. Level of expression of HGFR in itself is thus not necessarily associated with degree of ductal differentiation.

Since HGFR appears to be expressed by both benign and malignant epithelium, and is not restricted to duct-forming structures, (suggesting that the potential for HGF/HGFR binding is maintained in malignancy), it may be that functional differences

exist between benign and malignant breast tissue relating to HGF/HGFR. Such differences could include the activity of the ligand or receptor itself, and post-receptor signal transducer/effector systems. For example, cellular responsiveness to HGF has been shown to be influenced by factors (eg. urokinase) regulating HGF activation (42), or other extracellular mediators (eg. TGF- β), or extracellular matrix components (eg. heparan sulfate) (43,44). Alternatively, HGFR activation in benign epithelium may, as suggested by Tsarfaty *et al.* (2), result in activation of a morphogenic programme leading to duct formation. In malignancy, defective regulation of, or alteration of components of this programme (for example, loss of proper cell-cell adhesion) may result in an incomplete or "dysfunctional" programme. In fact, "scatter activity" of HGF on invasive carcinomas has been found to be influenced by the integrity of the cadherin system (eg. 45-47). Decreased expression of E-cadherin in more poorly differentiated carcinomas may render them more susceptible to the scatter activity of HGF. Thus, whereas HGF may induce ductal morphogenesis in the presence of an intact cadherin system in areas of well-differentiated carcinoma, disruption of cadherin expression in more poorly-differentiated areas may instead result in disaggregation, "scatter" and more invasive behaviour. Such a difference in pattern of response to HGF in different areas within a given tumor or by a given tumor at different times (during progression) could also explain what appears at first glance to be contradictory evidence in the literature for "tumor suppressing" vs. "tumor promoting" effects of HGF ligand and receptor.

Zonal co-expression of HGF ligand and receptor mRNA was also found in both benign and malignant epithelium, and would suggest the possibility of an HGF autocrine loop. This finding is consistent with recent evidence that HGFR expression may be upregulated by HGF (48). A similar autocrine loop effect for HGF and HGFR in malignant (and some non-malignant, proliferative) epithelial cells has been described previously in other systems (13-15). The higher levels of co-expression of ligand and receptor observed in ductal epithelial hyperplasia suggests that HGF may act in an autocrine role in non-neoplastic ducts as well, especially those undergoing benign proliferation. In the malignant situation, a normal reactive mechanism may be altered as suggested above, such that autocrine stimulation itself is no longer properly regulated, or the response to autocrine stimulation is altered, with the end result of invasion and metastasis ("malignant conversion") rather than morphogenesis and ductular differentiation ("tumor suppression"). Also in keeping with this hypothesis is the finding that in the majority of the tumors examined, highest levels of expression of both HGF and HGFR mRNA were found at the advancing margins of the tumor, where active invasion is taking place. This pattern of expression has been reported for other peptides, e.g. transforming growth factor β (49), platelet-derived growth factor β receptor (50), cathepsin B (51), and plasminogen activator (52), which may be involved in invasiveness at the tumor front. Interestingly, urokinase-type plasminogen activator is also an activator of HGF (42).

Further work (both *in vivo* and *in vitro*) is necessary to elucidate the role of HGF in the control of these aspects of epithelial cell behavior, and to study possible

alterations of the response to HGF in malignancy. The recent evidence in the literature for a strong potential prognostic role for the level of HGF expression (32) adds an extra level of significance and urgency to unravelling the role of HGF in human breast carcinoma.

ACKNOWLEDGEMENTS

We thank Dr. Iain Young for helpful suggestions regarding ISH with digoxigenin-UTP. We thank Mr. Nader Rahimi for helpful discussions in preparation of the manuscript. We also thank Mr. Lloyd Kennedy for artistic and photographic assistance, and Ms. Marg Morrow for help with the immunohistochemistry.

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Figure 1

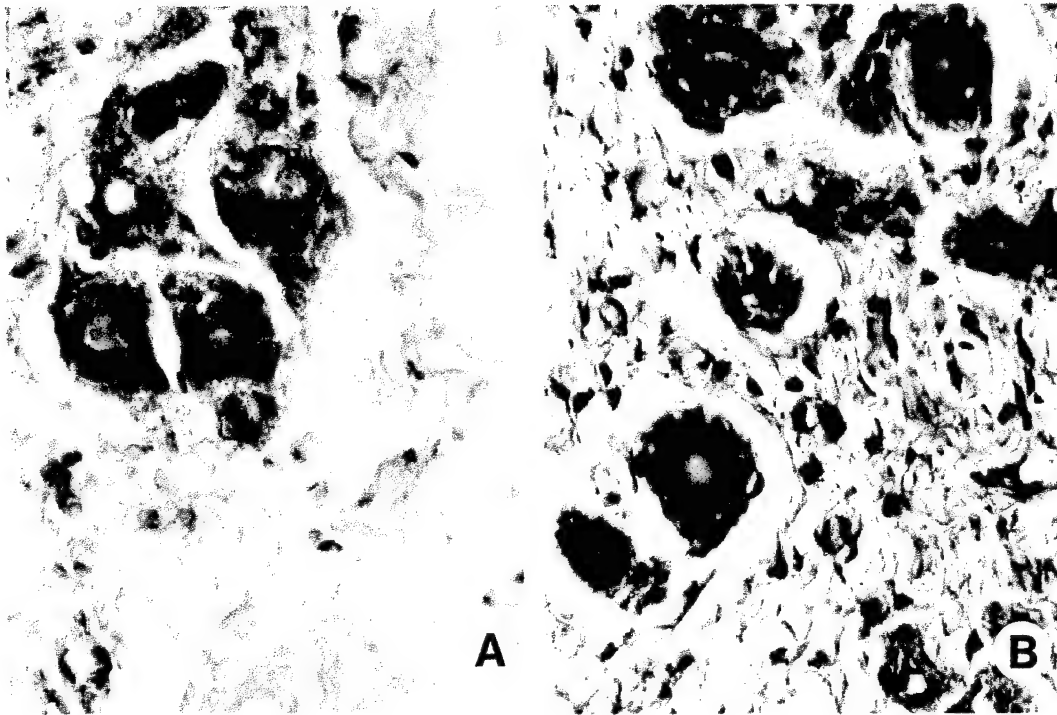


Figure 1: Non-malignant breast tissue A: ISH for HGFR mRNA showing a strong cytoplasmic brown-purple staining in ductal epithelial cells, but negligible cytoplasmic staining of stromal cells (Original magnification, X630). B: ISH for HGF mRNA showing variable cytoplasmic staining of benign epithelium, with focal areas of strong positivity. Some cytoplasmic staining is also apparent in stromal fibroblasts and endothelial cells (Original magnification, X630).

Figure 2

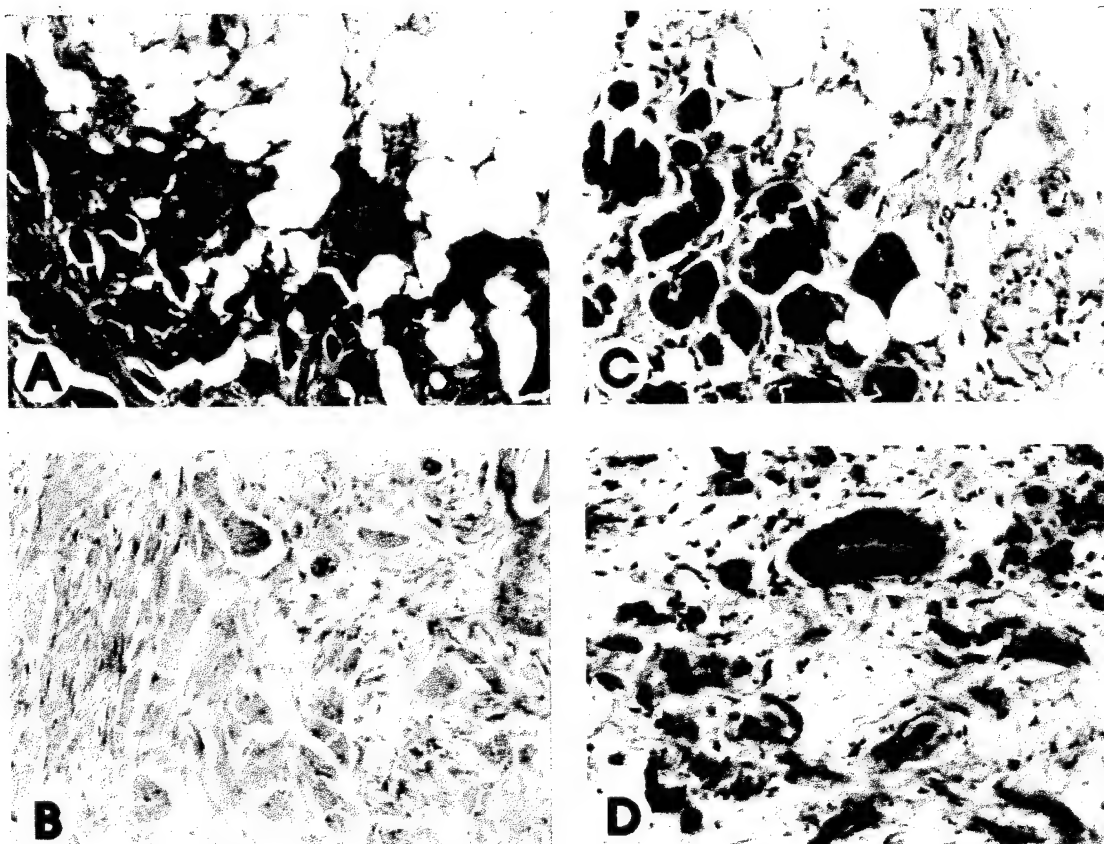


Figure 2: Moderately (A-C) and well (D) differentiated ductal carcinomas A: ISH for HGFR mRNA showing intense cytoplasmic positivity at the advancing margin of the tumor (Original magnification, X630). B: ISH for HGFR mRNA showed much weaker staining in the malignant epithelium in more central regions of the same tumor (Original magnification, X250). C: ISH for HGF mRNA showing strong cytoplasmic positivity of the malignant epithelium at the advancing margin of the same tumor, in the same region which stained intensely for HGFR mRNA (Original magnification, X630) (cf. Fig. 2A). In addition, stromal fibroblasts and endothelial cells showed some cytoplasmic positivity. D: ISH analysis for HGFR mRNA showing IDC surrounding a benign duct. Intensity of cytoplasmic staining is similar in both benign and malignant epithelium, regardless of whether the malignant epithelium forms well-defined ductular structures (Original magnification, X400).

Figure 3

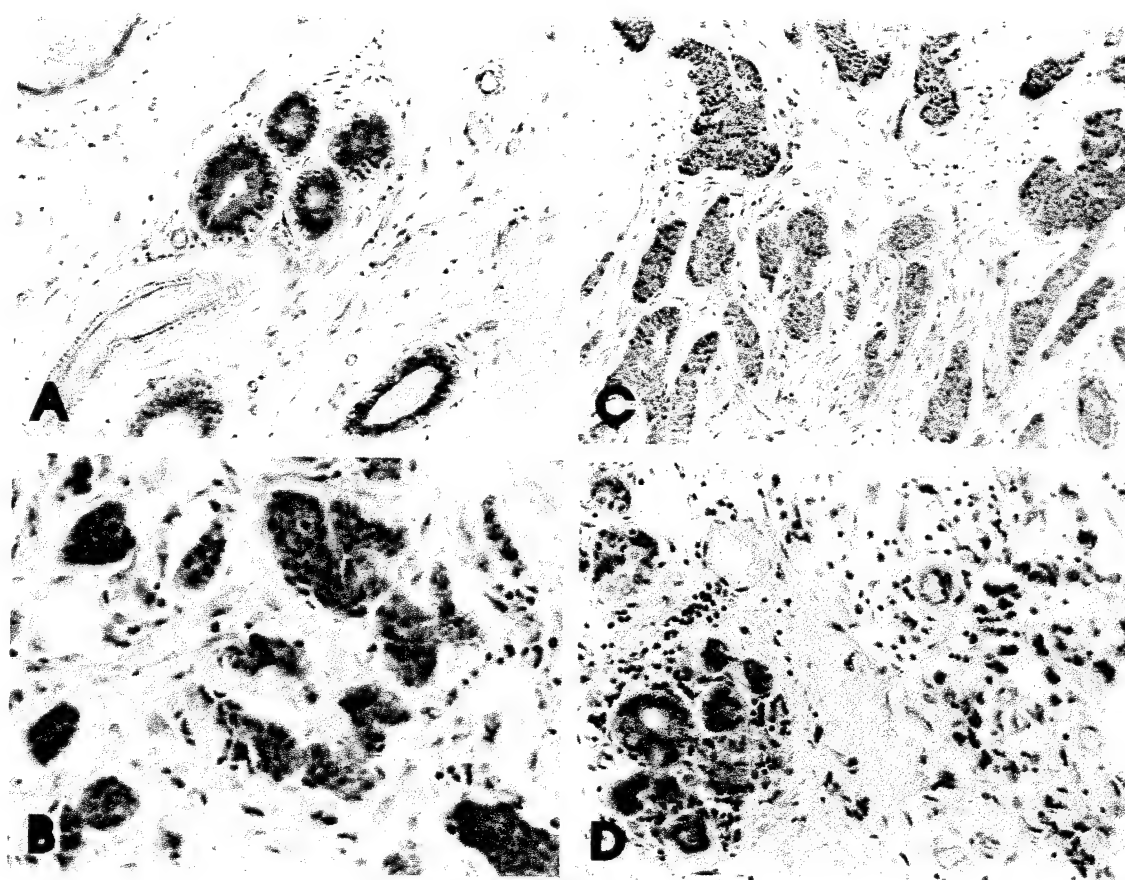


Figure 3: Immunoperoxidase localization of HGFR protein expression A: Benign ducts showing strong cytoplasmic/membrane positivity (brown) (original magnification, X250). B: Moderately differentiated IDC showing strong cytoplasmic/membrane positivity at the advancing margin (original magnification, X250). C: Same tumour as in B, showing gradient of staining intensity: strongest closer to the advancing margin (top), and weakest in more central regions (bottom) of the tumour (original magnification, X250). D: Poorly differentiated IDC (right) adjacent to non-malignant ducts (left) showing comparable staining intensity in both malignant (non-duct-forming) and non-malignant (ductal) epithelium (original magnification, X400).

IDENTIFICATION OF A HEPATOCYTE GROWTH FACTOR AUTOCRINE LOOP IN A MURINE MAMMARY CARCINOMA¹

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ABSTRACT:

Constitutive activation of growth factor receptors through autocrine/paracrine mechanisms occurs frequently in human cancers and is thought to play an important role in carcinogenesis. We have previously demonstrated that hepatocyte growth factor (HGF)³ is a potent mitogenic factor for murine mammary carcinoma (SP1) cells *in vitro*. We report here an autocrine HGF loop in SP1 cells. HGF receptor/Met is expressed in SP1 cells and is constitutively tyrosine-phosphorylated. The phosphorylation of HGF receptor/Met is inhibited when cells are exposed to suramin or anti-HGF IgG. This finding suggests that constitutive tyrosine-phosphorylation of HGF receptor/Met is sustained by an extracellular factor, most likely HGF. Using northern blot and western blot analysis, we detected expression of a 6 kb HGF mRNA in SP1 cells and an 85 KDa HGF protein in SP1 conditioned medium, respectively. *In vitro* translation of mRNA from SP1 cells and metabolic labeling confirmed expression and synthesis of HGF by SP1 cells. SP1 cells also invade through matrigel-coated transwell membranes in an *in vitro* invasion assay and invasion of these cells was inhibited by neutralizing anti-HGF IgG. In addition, SP1 conditioned medium induced scatter activity of Madin-Darby canine kidney epithelial cells, and this activity was inhibited by neutralizing anti-HGF IgG. We have also shown that several signaling molecules including phosphatidylinositol 3-kinase, Src, focal adhesion kinase, and phospholipase C- γ in SP1 cells are constitutively tyrosine-phosphorylated, suggesting that co-expression of HGF and HGF receptor/Met may in part contribute to sustained tyrosine-phosphorylation of these cytoplasmic proteins in SP1 cells. Our observations in the SP1 model suggest that HGF contributes to growth and invasive phenotypes of mammary carcinomas via both paracrine and autocrine mechanisms.

RUNNING TITLE:

HGF Autocrine Loop in Breast Cancer

FOOTNOTES:

- 1) This work was supported by grants from the National Cancer Institute of Canada and the U.S. Army Materiel and Command (No. DAMD 17-94-J-4407).
- 2) To whom correspondence should be addressed.
- 3) List of Abbreviations: CM, conditioned medium; FBS, fetal bovine serum; FAK, focal adhesion kinase; HGF, hepatocyte growth factor; PI, phosphatidylinositol; PLC- γ , phospholipase C- γ ; PY, phosphotyrosine; PKC, protein kinase C; PBS, phosphate buffered saline.

INTRODUCTION:

Aberrant expression of growth factors and their receptors has been shown to be associated with many types of human cancers (1,2,3,4), and in some instances deregulation in growth factor-mediated signaling pathways may contribute to the malignant transformation of human cancers. It is known that such aberrant expression may be due to structural alteration of the corresponding growth factor receptors (2,5), or to the creation of an autocrine loop (6,7,8,9). Recently, attention has focused on the role of hepatocyte growth factor (HGF) receptor/Met in human cancers. HGF is a heterodimeric cytokine with molecular weight of 85 KDa (10), and exhibits pleiotropic biological functions as a mitogen, motogen, morphogen and angiogenic factor (11,12). HGF has been detected primarily in fibroblasts and other mesenchymal/stromal cells, and acts on epithelial cells in a paracrine mechanism (13,14,15,16). However, it has been shown that HGF (17) or a variant of HGF (18,19), produced by non-differentiated human keratinocytes (17), human normal bronchial epithelial and lung carcinoma cells (18,19), may act as an autocrine factor in these cells.

In contrast to its ligand, the HGF receptor/Met, a product of the *met* proto-oncogene (20,21), is expressed most prominently in epithelial cells (22,23). The HGF receptor/Met protein is a heterodimer of 190 KDa composed of a 145 KDa β , and a 50 KDa α , subunit. The β -subunit extends through the membrane and contains the tyrosine kinase catalytic domain (24,25). There is also evidence that the *met* proto-oncogene is over-expressed (22,23,26,27,28), and amplified (23) in a significant number of human carcinomas. In some cases, constitutive activation of HGF receptor/Met was also observed (29). Recently, our laboratory has shown that HGF and HGF receptor/Met mRNA are co-expressed by human breast carcinoma cells *in situ*, particularly in

regions of invasive carcinoma (30), suggesting a possible autocrine HGF loop in human breast cancer. However, the existence of an HGF autocrine loop in breast carcinomas, and its putative role in growth and metastasis of breast cancer is not known.

To better understand the possible paracrine and autocrine role of HGF in the growth of mammary carcinomas, we have previously developed a model to examine the influence of stromal cells, particularly adipocytes, on the growth of a murine mammary carcinoma, SP1 (31). We have subsequently shown that a fully differentiated 3T3-L1 adipocyte cell line secretes HGF, which stimulates growth of SP1 carcinoma cells by a paracrine mechanism (32). In the present study, we demonstrated that HGF and HGF receptor/Met are co-expressed in SP1 cells. We therefore examined the activation of HGF receptor/Met and the functional activity of secreted HGF in SP1 cells. Our observations in the SP1 model suggest that HGF may contribute to continuous growth and invasiveness of mammary carcinomas via paracrine and autocrine mechanisms.

RESULTS:

Constitutive tyrosine-phosphorylation of HGF receptor/Met in SP1 cells :

As a first step to assess the presence of an operative HGF autocrine loop in breast carcinomas, we determined the tyrosine-phosphorylation status of HGF receptor/Met expressed in the mammary carcinoma, SP1. Using immunoprecipitation and western blotting of proteins from SP1 cell lysates with anti-Met IgG directed to the carboxyl terminus of HGF/Met receptor, we showed that SP1 cells express a p170^{met} (Met precursor) and p145^{met} (β-chain of mature HGF receptor/Met) (Figure 1A). SP1 cells also express a 110 KDa protein that was immunoprecipitated with anti-Met IgG. The 110 KDa protein may correspond to an alternative variant of HGF receptor/Met or enzymatic cleavage of p145^{met} (this possibility is currently being investigated). Immunoprecipitation of all three HGF receptor/Met proteins was inhibited by co-incubation of cell lysates with a peptide corresponding to the carboxyl terminus of HGF receptor/Met (32). Reprobing of the same membrane with anti-PY IgG showed that p145^{met} and p170^{met} are tyrosine-phosphorylated in SP1 cells incubated without exogenous HGF, and the level of tyrosine-phosphorylation of these proteins increased following incubation of SP1 cells with exogenous HGF (Figure 1B). In addition, a metastatic variant of SP1, SP1-3M, showed constitutive tyrosine-phosphorylation of p145^{met} (Figure 1C). These results suggest that constitutive tyrosine-phosphorylation of HGF/Met receptor in these cells may be sustained by an extracellular factor, most likely HGF protein.

Tyrosine-phosphorylation and activation of signal transducers in SP1 cells: Receptor tyrosine kinases such as HGF receptor/Met, upon activation by ligand, undergo autophosphorylation due

to activation of their intrinsic tyrosine kinase activities. In addition, activated HGF receptor/Met associates with, and phosphorylates, a number of cytoplasmic proteins that contain SH2 domains, including phospholipase C- γ , Src, Shc, and the p85 subunit of phosphatidylinositol (PI) 3-kinase (33). Since HGF receptor/Met is constitutively tyrosine-phosphorylated in SP1 cells, we therefore investigated whether some of these signal transducers are also tyrosine-phosphorylated in SP1 cells (Figure 2). Proteins from lysates of serum-starved SP1 cells were immunoprecipitated with anti-PLC- γ , anti-PI 3-kinase, anti-FAK or anti-Src antibodies; immune complexes were subjected to SDS-PAGE under reducing conditions (except for Src, see Figure 2, legend) and immunoblotted with anti-PY antibody. The results showed that all four signal transducer molecules are tyrosine-phosphorylated in serum-starved SP1 cells without exogenous growth stimulator. However, the cytokine-activated signaling proteins JAK1, JAK2, JAK3, STAT1 (34) or the EGF receptor substrate, Eps15 (35), though present, were not detected in a tyrosine-phosphorylated form in SP1 cells (data not shown). These results further support the presence of an operational autocrine loop in SP1 cells, and suggest that signaling pathways stimulated by growth hormone, prolactin, erythropoitin, interleukins-1,-2,-4,-9, interferons, or EGF are not operating in SP1 cells in an autocrine manner.

Inhibition with suramin and anti-HGF IgG of tyrosine-phosphorylation of HGF receptor/Met in

SP1 cells: Detection of constitutive tyrosine-phosphorylation of HGF/Met receptor in SP1 cells prompted us to perform experiments to interfere with such a putative autocrine activation of HGF/Met receptor in these cells. Preliminary results showed that treatment of serum-starved SP1 cells with erbastatin, a tyrosine kinase inhibitor, or low pH (3.7), a condition that has been shown

to be effective in dissociating growth factor ligand/receptor complexes (36,37), caused dephosphorylation at tyrosine residues of HGF receptor/Met in a time-dependent and reversible process (data not shown). We now show that constitutive tyrosine-phosphorylation of HGF receptor/Met was significantly decreased in a time-dependent manner by suramin, a known blocker of autocrine loops (29,38) (Figure 3A). In addition, tyrosine-phosphorylation of HGF receptor/Met was decreased by treatment of cells with anti-HGF IgG (Figure 3B). It should be pointed out that anti-HGF IgG treatment alone had little effect on tyrosine-phosphorylation of HGF receptor/Met at the concentration used (10-30 $\mu\text{g/ml}$). However, when serum-starved SP1 cells were washed with 0.5 M NaCl and RPMI medium (pH 3.7) to remove extracellular HGF and subsequently incubated with anti-HGF IgG (100 $\mu\text{g/ml}$) for 4 h, a sustained dephosphorylation of HGF receptor/Met was observed; in contrast, cells incubated in parallel cultures with non-immune IgG showed no change in tyrosine-phosphorylation of HGF receptor/Met (Figure 3B). Together, these data suggest that constitutive tyrosine-phosphorylation of HGF receptor/Met in SP1 cells may be sustained by an extracellular factor, most likely HGF ligand, and that dephosphorylation of the HGF receptor/Met can be effectively achieved by dissociation of the HGF ligand/HGF receptor complex.

Detection of HGF mRNA in SP1 cell lysate and of HGF protein and functional activity in SP1 conditioned medium (CM): Detection of constitutively active HGF/Met receptor in SP1 cells prompted us to investigate expression of HGF in SP1 cells. We therefore evaluated the expression of HGF in SP1 cells both at mRNA and protein levels. Poly(A)⁺ mRNA was extracted from SP1 cells, and subjected to northern blot analysis with a mouse HGF cDNA probe. We found that SP1

cells express a 6 kb HGF mRNA transcript (Figure 4). In addition, total mRNA from SP1 cells was subjected to *in vitro* translation and assayed for the presence of HGF using western blot analysis. A single band with molecular weight of 80 KDa was detected (Figure 5A). Western blot analysis of conditioned medium (CM) from SP1 and SP1-3M cells with anti-HGF IgG also confirmed the presence of a protein with molecular weight of 85 KDa in non-reducing conditions and 69 KDa in reducing conditions, corresponding to the mature and α -subunit of HGF protein, respectively (Figure 5B). To analyse further the synthesis of HGF by SP1 cells we performed metabolic pulse-chase labeling experiments. Pulse-chase analysis and immunoprecipitation with anti-Met IgG showed a protein with molecular weight of 85 KDa (Figure 5C). A longer exposure showed an additional protein with apparent molecular weight of 110 Kda (data not shown). The latter band may correspond to the pro-HGF protein.

To assess functional activity of SP1 tumor-derived HGF, we evaluated the involvement of the secreted HGF in cell invasion, growth and scatter. Invasion of SP1 cells was measured in a transwell and was expressed as the relative number of cells that penetrated the matrigel-coated membrane during the course of the assay (Table 1). SP1 cells showed significant spontaneous invasion in low-serum (0.1%) medium; this invasion was strongly inhibited by anti-HGF IgG and was enhanced by exogenous HGF. In addition, SP1 cells grew continuously in serum-free or low-serum medium, and this growth was inhibited by suramin (data not shown). It is noteworthy that two different preparations of anti-HGF IgG and non-immune IgG were mitogenic for SP1 cells, and therefore provided no definitive information about a role of HGF in spontaneous growth of SP1 cells. It is possible that our inability to inhibit growth of SP1 cells with anti-HGF IgG is due to presence of growth promoting agents in the sample or IgG itself is mitogenic for these

cells, since normal rabbit IgG, normal turkey IgG, normal horse IgG all showed variable mitogenic effects on SP1 cells. Whatever the case, these findings suggest that both HGF and HGF receptor/Met are expressed by SP1 cells and that growth and invasive phenotypes of SP1 cells may be facilitated by an autocrine growth factor loop. We have therefore independently evaluated the biological activity of HGF secreted by SP1 cells by assaying SP1 CM for stimulation of scatter activity of Madin-Darby canine kidney epithelial (MDCK) cells. SP1 CM induced a strong scatter activity of MDCK cells, compared to RPMI medium alone (Figure 6A, 6B). The identification of HGF in SP1 CM as the scatter-inducing factor was confirmed by the inhibition of scatter activity of SP1 CM by neutralizing anti-HGF IgG (Figure 6D), whereas control IgG had no effect (Figure 6C).

DISCUSSION:

In this paper we have presented evidence for the presence of an autocrine HGF loop in SP1 mammary carcinoma cells on the basis of three criteria: First, SP1 cells express constitutively-activated HGF receptor/Met and this activity is significantly inhibited by dissociation of HGF ligand from HGF receptor/Met. Second, a 6 kb HGF mRNA and an 85 KDa HGF protein, corresponding to the reported sizes of HGF mRNA and mature protein (10), respectively, are produced by SP1 cells. Third, SP1 CM induces strong invasion of SP1 cells and strong scatter activity of MDCK cells; induction of these functions is inhibited by anti-HGF IgG.

Evidence supports the involvement of different signal transduction pathways in HGF-

dependent functions (33). We have demonstrated constitutive tyrosine-phosphorylation of PLC- γ , p85 subunit of PI 3-kinase, and FAK in SP1 cells. These results suggest that creation of an HGF autocrine loop in SP1 cells induces specific signaling pathways, some of which may contribute to proliferation and invasion of these cells. However, the cytokine-activated signaling proteins JAK1, JAK2, JAK3, STAT1 (34) or the EGF receptor substrate Eps15 (35), though present, were not tyrosine-phosphorylated in SP1 cells (data not shown). Thus interleukin-1,-2,-4,-9, and EGF receptor-mediated signaling pathways are not activated in an autocrine manner in SP1 cells under the culture conditions used. Whether cooperative stimulation via other growth factors or extracellular matrix components contributes to autocrine activation of signaling molecules in SP1 cells remains to be investigated.

Autocrine growth factors appear to be important in malignancy and metastasis. Many types of tumors release growth factors, including transforming growth factor- α , platelet-derived growth factor, bombesin and transforming growth factor- β ; these growth factors can act in an autocrine manner (8). Furthermore, an autocrine mechanism of transformation has been demonstrated for a variety of growth factors including v-sis, colony stimulating factor-1, and interleukin-3 (39,40,41). HGF and HGF receptor/Met might also play a significant role in tumor development and growth. This view was strongly supported by the fact that transfection of a full size *met* cDNA into NIH 3T3 fibroblasts results in establishment of an HGF autocrine loop, and induces transformation and tumor growth of these cells in nude mice (42). Furthermore, the *met* proto-oncogene was found to be over-expressed in a significant number of human cancers of epithelial origin (22,23,26,27,28). Experiments are in progress to determine the possible role of an HGF autocrine loop in growth and metastasis of breast carcinoma cells.

The clinical import of HGF in human breast cancer was highlighted recently in the work of Yamashita *et al.* (43,44) who reported that a high level of expression of HGF is an even more significant factor in predicting poor relapse-free and overall survival than is lymph node status. However, what cells produce HGF in breast tumors and the potential role of HGF in breast cancer is not known. Tsarfaty *et al.* (45) have reported that HGF receptor/Met protein is expressed in both benign and malignant breast epithelium and that various breast cancer cell lines respond to HGF. Wang *et al.* (46) reported that HGF mRNA is expressed in both benign and malignant mammary epithelium, and that the most abundant expression in benign epithelium was in regions of proliferative activity. Our laboratory (30) has recently shown co-expression of HGF and HGF receptor/Met mRNA in benign and malignant epithelium; expression was particularly intense at the migrating tumor front. Together these results support a possible autocrine role of HGF in human breast cancer.

We have previously demonstrated that adipocytes, a dominant stromal cell type in glandular tissues, support growth of SP1 cells, and subsequently we have reported that HGF is the major mitogenic factor for SP1 cells in 3T3-L1 adipocyte CM (32). These findings suggest a paracrine role for HGF in breast cancer (32). Recent data from Wang *et al.* (46) and our laboratory (30) support a possible autocrine role of HGF in human breast carcinomas *in situ*. Our present results provide further support of an HGF autocrine loop in the mammary carcinoma cell line, SP1. Therefore, co-expression of HGF and HGF receptor/Met may provide a selective advantage for the progression of mammary carcinoma cells toward a more aggressive phenotype, making them independent of their surrounding tissues. Our present report indicates that both paracrine and autocrine HGF loops exist in SP1 mammary carcinoma cells and provides a

physiologically-relevant model for assessing the role of paracrine and autocrine pathways for HGF in breast cancer development.

MATERIAL AND METHODS:

Reagents: Monoclonal mouse anti-phosphotyrosine (PY) antibody was purchased from Transduction Laboratories. Polyclonal rabbit anti-Src, anti-PI 3-kinase, and anti-FAK IgG were purchased from UBI. Polyclonal rabbit anti-PLC- γ was kindly provided by C. Ellis (47). Polyclonal rabbit anti-murine Met IgG was prepared as described (48) and sheep anti-human HGF IgG (which cross-reacts with the α -subunit of mouse HGF) was obtained from Genentech Inc. Enhanced chemiluminescence (ECL) reagent was from Amersham. Prestained molecular weight standards were from GIBCO. Suramin was from FBA Pharmaceutical Inc.

Cell Culture: The SP1 tumor cell line is a spontaneous non-metastatic murine mammary intraductal adenocarcinoma isolated from an 18 month old CBA/J female. The characteristics of the SP1 cell line have been described elsewhere (49). SP1-3M cells are a metastatic variant cell line derived from SP1 cells (31). Maintenance medium for SP1 and SP1-3M cells was RPMI (GIBCO) supplemented with 20 mM L-glutamine and 7% fetal bovine serum (FBS, GIBCO).

Scatter Assay: Scatter assays were performed as previously described (18). In brief, SP1 CM (10 ml), concentrated to 1 ml using micro-concentrating Centricon-30 tubes (Amicon), was added to MDCK cells, which had been plated overnight in DMEM plus 5% FBS at 3×10^3 cells per well

in 24-well Linbro plates. After a 24 h incubation, the degree of scattering was assessed visually. Photographs were taken using a Leica inverted microscope with Kodak Technical Pan film.

Invasion assay: Invasion assays were performed in transwells (Corning Costar) with a 6.5 mm diameter polycarbonate filter of 8 μ m pore size, as described previously (50). Filters were pre-coated with matrigel (Collaborative Research) (20 μ g/ml in RPMI), and dried overnight. SP1 cells (1×10^4) in 200 μ l 0.2% FBS/RPMI were added to the upper well and 800 μ l various media as indicated were added to the lower well. After incubation for 36 h at 37°C, transwells were removed and the tumor cells fixed with 1% glutaraldehyde in phosphate buffered saline (PBS) for 20 min. Cells were stained with hematoxylin, and the cells and matrigel on the upper surface were removed with a cotton swab. Filters were dried and the number of cells in a constant central area of each well was assessed using an inverted light microscope. In this way, cells that had invaded through the membrane could be directly assessed.

Immunoprecipitation and Immunoblotting: SP1 cells were grown to confluency and serum-starved for 24 h. Cells were rinsed with cold PBS buffer three times and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% NP-40, 1 mM sodium orthovanadate, 50 mM NaF, 2 mM EDTA, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 mM PMSF, and 1 μ M pepstatin. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. The supernatants were incubated with 5 μ l rabbit anti-Met IgG or the indicated antibodies at 4°C for 1 h. Immunoprecipitates were collected on protein-A sepharose, washed three times with lysis buffer, separated on 8% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA, and probed with anti-PY (1:500), or anti-

Met (1:200) antibodies. The membrane was washed four times with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 h, incubated with horse-radish peroxidase-labeled secondary anti-rabbit or anti-mouse IgG antibodies (1:5000) for 1 h, and washed with TBST, three times. Immune complexes were detected using ECL.

RNA extraction and northern analysis: The Micro-Fast Track system (InVitrogen) was used to extract poly (A)⁺ RNA from cultured cells. mRNA was subjected to denaturing electrophoresis on a 1.4% agarose gel, then transferred to Zeta-Probe Membranes (Bio-Rad). The hybridization method used was a modification of that of Sambrook *et al.* (51): Blots were pre-hybridized for 4 hours at 42°C in standard hybridization solution (50% formamide, 4x Denhardt's solution, 0.25 x SSC, 0.1 mg/ml sheared herring testes DNA, 1% SDS, 5 mM EDTA, 50 mM NaH₂PO₄). ³²P-labeled probes were prepared by a nick translation method (Nick Translation Kit, GIBCO) and hybridized with the membrane for 24 h at 42°C in standard hybridization solution. Membranes were washed for 15 min at room temperature with each of the following: 2x SSC/0.1% SDS, 0.5x SSC/0.1% SDS and 0.1x SSC/0.1% SDS. The cDNA probe for HGF was obtained from T. Nakamura (10).

In vitro Translation: Poly (A)⁺ was extracted from SP1 cells and *in vitro* translation was carried out by using a rabbit reticulocyte system (Amersham). The resulting polypeptide products were subjected to western blot analysis.

Metabolic Labeling and Immunoprecipitation: SP1 cells were metabolically labeled for 18 h with [³⁵S]-methionine (Dupont) at 50 μ Ci/ml in methionine-free RPMI medium (GIBCO). CM was concentrated and immunoprecipitated with anti-HGF IgG. Immune complexes were analysed by SDS-PAGE, fluorographed, dried and exposed to film for autoradiography.

ACKNOWLEDGEMENTS:

We thank Dr. T. Nakamura for providing HGF cDNA, and Dr. P. Greer for his critical comments during the preparation of this manuscript.

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TABLE 1

**AUTOCRINE STIMULATION OF CELL INVASION IN SP1 CELLS IS
INHIBITED BY ANTI-HGF IgG**

Conditions ^a	Relative No. of Cells Invaded Per Well ^b
SP1 CM	24.6 ± 12.9
SP1 CM + anti-HGF IgG	3.0 ± 2.6 ^c
rHGF	70.0 ± 10

Legend:

- a) Invasion assays were performed in matrigel-coated transwells as described in Materials and Methods. SP1 conditioned medium with or without polyclonal rabbit anti-HGF IgG (15 µg/ml), or rHGF (20 ng/ml) was added to the lower well (800 µl). SP1 cells (10⁴) were added in 200 µl RPMI with 0.2% FBS to the upper well. Cells that had initiated invasion were stained and counted on the underside of the membrane after 36 h.
- b) Values represent the mean ± S.D. of at least 3 wells per group.
- c) Asterisk indicates significant reduction in the number of invading cells compared to the group with SP1 CM alone (p=0.0296). A two-tailed Student T-test was used. No difference between medium alone, SP1 CM, without or with non-immune IgG, was observed (data not shown).

FIGURE LEGENDS:

FIGURE 1: Tyrosine-phosphorylation of HGF receptor/Met in SP1 cells, untreated or treated with exogenous HGF: Non-metastatic SP1 cells were serum-starved for 24 h, were incubated without (-) or with (+) HGF for 20 min, and were lysed as described in Materials and Methods.

Panel A: Proteins from SP1 cell lysates were immunoprecipitated with anti-Met IgG, separated on 8% SDS-PAGE under reducing conditions, and subjected to western blot analysis. The nitrocellulose membrane was immunoblotted with anti-Met IgG. Immune complexes were detected by ECL. Protein molecular weight standards are shown on the right.

Panel B: The same nitrocellulose membrane was stripped of antibodies and reprobed with anti-PY antibody.

Panel C: Metastatic SP1-3M cells were serum-starved, lysed as in Panel A and immunoprecipitated with anti-Met IgG, nonimmune IgG or anti-PY, separated by SDS-PAGE under reducing conditions and blotted with anti-Met IgG.

FIGURE 2: Tyrosine-phosphorylation state of Src, PI 3-kinase, PLC- γ and FAK in SP1 cells:

SP1 cells were serum-starved for 24 h. Proteins from SP1 cell lysates were immunoprecipitated with polyclonal rabbit anti-PLC- γ , anti-FAK, anti-PI 3-kinase or anti-Src IgG, and separated on 8% SDS-PAGE under reducing conditions (except for Src which requires non-reducing conditions since Src and IgG heavy chains have similar molecular weights). Proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-PY antibody. Immune complexes were

detected by ECL. Protein molecular weight standards are shown on the right. Distinct proteins, p150, p125, p85, and p60 were detected in each track, corresponding to the known M_r of PLC- γ , FAK, p85 subunit of PI 3-kinase, and Src, respectively.

FIGURE 3: Effect of suramin or anti-HGF IgG on tyrosine-phosphorylation state of HGF receptor/Met in SP1 cells:

Panel A: SP1 cells were serum-starved for 24 h and incubated in 100 μ M suramin for the indicated times, lysed and immunoprecipitated with anti-Met IgG. Immunoprecipitates were separated on 8% SDS-PAGE under reducing conditions and subjected to western blot analysis. The nitrocellulose membrane was immunoblotted with anti-PY antibody. Immune complexes were detected by ECL. Protein molecular weight standards are shown on the right.

Panel B: Serum-starved SP1 cells were washed with 0.5 M NaCl and RPMI medium (pH 3.7), and subsequently incubated with medium alone (Control), non-immune IgG or anti-HGF IgG (each at 100 μ g/ml) for 4 h. Immunoprecipitation and western blotting was carried out as in Panel A.

FIGURE 4: Detection of HGF mRNA in SP1 cells: Poly-A⁺ RNA extracted from SP1 cells was subjected to gel electrophoresis (1 μ g per lane), transferred to Zeta probe membrane and hybridized with an HGF cDNA probe. A 6 kb HGF mRNA was evident. A probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to evaluate relative amounts of mRNA transferred to the membrane.

Figure 5: Detection of HGF protein in SP1 cells: **Panel A:** *In vitro* translation of HGF RNA transcript: Poly (A)⁺ RNA was extracted from SP1 cells, translated in a rabbit reticulocyte system and analyzed by western blotting. None, SP1, and Control correspond to: no added RNA, 2 μ g RNA from SP1 cells, and unrelated RNA supplied by Amersham as a negative control, respectively. The membrane was probed with polyclonal rabbit anti-HGF IgG and protein bands were detected by ECL.

Panel B: SP1 CM and SP1-3M CM (25 ml each) were concentrated in Centricon-30 tubes, electrophoresed through 10% SDS-PAGE under non-reducing or reducing conditions as indicated, and blotted onto nitrocellulose membrane. The membrane was probed with polyclonal rabbit anti-HGF IgG.

Panel C: Metabolic labeling of SP1 cells: Cells (5×10^6) were metabolically labeled with [³⁵S]-methionine for 18 h as described in Materials and Methods. CM from SP1 cells (10 ml) was immunoprecipitated with anti-HGF antibody, analyzed by SDS-PAGE and autoradiographed. Protein molecular weight standards are shown on the right.

FIGURE 6: Effect of SP1 CM on scatter activity of MDCK cells: MDCK cells (5000/well) were plated in 24 well Linbro plates and incubated at 37 °C for 24 hr in (A) SP1 CM, (B) RPMI medium, (C) SP1 CM plus control rabbit IgG, and (D) SP1 CM plus rabbit anti-HGF IgG (both IgGs were at 15 μ g/ml). SP1 CM was 10x concentrated using Centricon 30 tubes. Cells were viewed under a Leica inverted microscope and photographed with Kodak Technical Pan film (Original magnification, 100X).

Figure 1

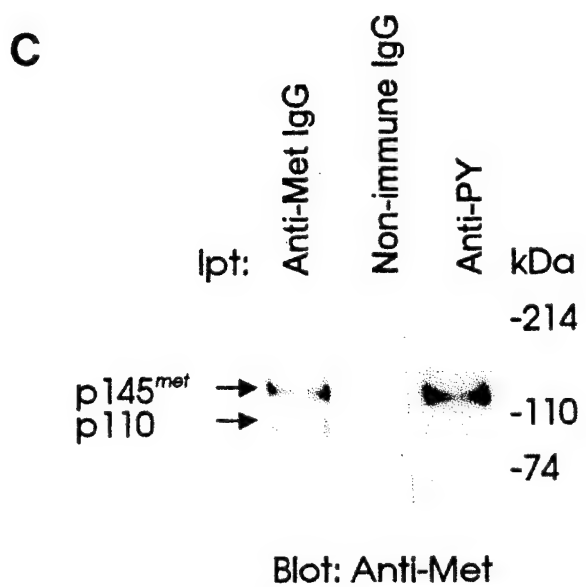
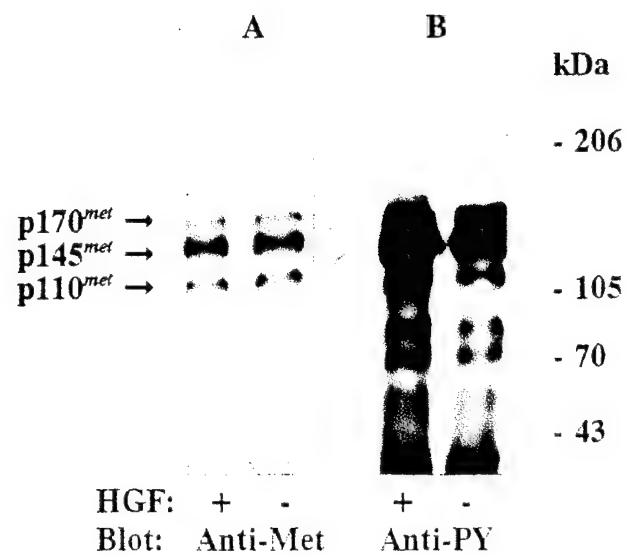
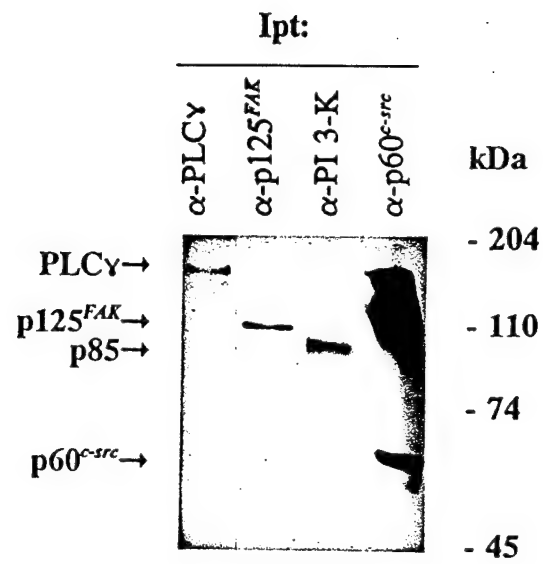


Figure 2



Blot: Anti-PY

Figure 3

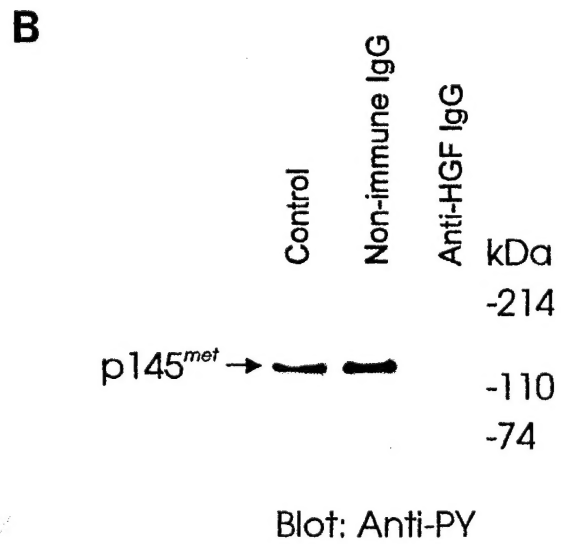
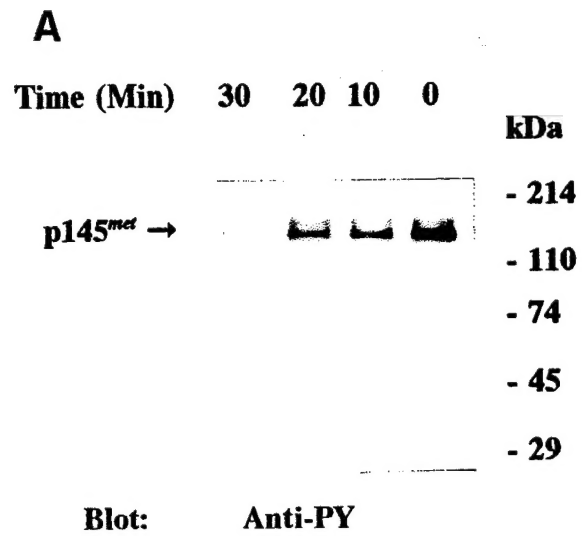


Figure 4

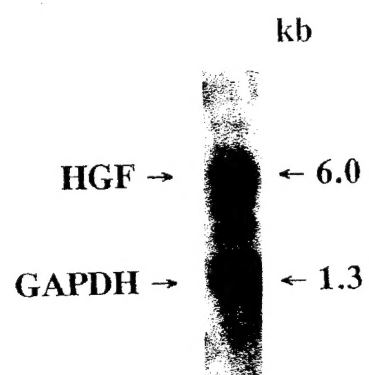
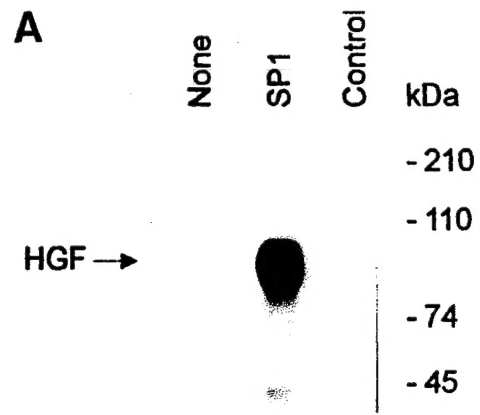
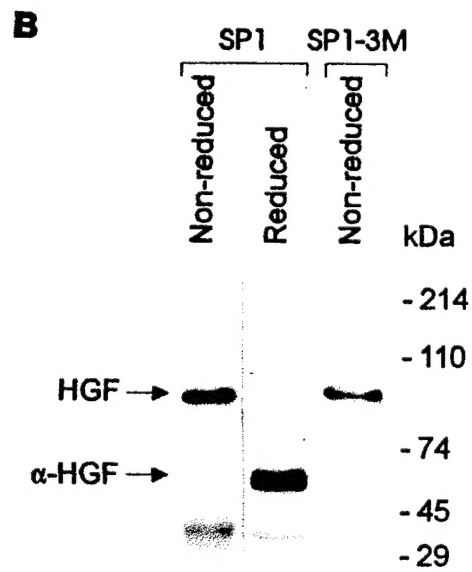


Figure 5



Blot: anti-HGF



Blot: Anti-HGF

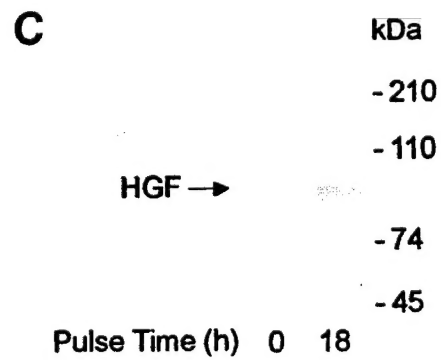


Figure 6

